BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

THIS PAGE BLANK (USPTO)

PCT ORLD INTELLECTUAL PROPERTY ORGANIZA
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C11D 3/386, C07K 19/00, C12N 9/00

A1

(11) International Publication Number: WO 97/28243

(43) International Publication Date: 7 August 1997 (07.08.97)

(21) International Application Number: PCT/DK97/00042

(22) International Filing Date: 29 January 1997 (29.01.97)

(30) Priority Data:
0094/96 29 January 1996 (29.01.96) DK

(71) Applicant *(for all designated States except US)*: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: VON DER OSTEN, Claus [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). CHERRY, Joel, R. [US/US]; 916 Anderson Road, Davis, CA 95616 (US). BJØRNVAD, Mads, Eskelund [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). VIND, Jesper [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). RASMUSSEN, Michael, Dolberg [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK).

(74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PROCESS FOR REMOVAL OR BLEACHING OF SOILING OR STAINS FROM CELLULOSIC FABRIC

(57) Abstract

The invention relates to a process for removal or bleaching of soiling or stains present on cellulosic fabric, wherein the fabric is contacted in aqueous medium with a modified enzyme (enzyme hybrid) which comprises a catalytically active amino acid sequence of a non-cellulolytic enzyme linked to an amino acid sequence comprising a cellulose-binding domain. The invention further relates to a detergent composition comprising an enzyme hybrid of the type in question and a surfactant, and to a process for washing soiled or stained cellulosic fabric, wherein the fabric is washed in an aqueous medium to which is added such a detergent composition.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

PROCESS FOR REMOVAL OR BLEACHING OF SOILING OR STAINS FROM CELLULOSIC FABRIC

FIELD OF THE INVENTION

The present invention relates to an improved enzymatic process for cleaning fabric or textile, notably cellulosic fabric or textile, particularly for removing or bleaching stains present on cellulosic fabric.

BACKGROUND OF THE INVENTION

Enzymatic processes for washing clothes (laundry washing) and other types of fabric or textile have been known for many years.

Certain types of soiling or stains have generally been found to be problematical to remove in such washing procedures. These are typically stains originating from starch, proteins, fats, red wine, fruit (such as blackcurrant, cherry, strawberry or tomato), vegetables (such as carrot or beetroot), tea, coffee, spices (such as curry or paprika), body fluids, grass, or ink (e.g. from ball-point pens or fountain pens).

It is an object of the present invention to improve the performance of a washing enzyme under conventional washing conditions by modifying the enzyme so as to alter (increase) the affinity of the enzyme for cellulosic fabric, whereby the modified enzyme is believed to be able to come into closer contact, and/or more lasting contact, with the soiling or stain in question.

SUMMARY OF THE INVENTION

It has now surprisingly been found possible to achieve improved cleaning of cellulosic fabric or textile, particularly improved removal or bleaching of stains present thereon, by means of an enzymatic process wherein the fabric or textile is contacted with an enzyme which has been modified so as to have increased affinity (relative to the unmodified enzyme) for binding to a cellulosic fabric or textile.

DETAILED DESCRIPTION OF THE INVENTION

The present invention thus relates, *inter alia*, to a process for removal or bleaching of soiling or stains present on cellulosic fabric or textile, wherein the fabric or textile is contacted in aqueous medium with a modified enzyme (enzyme hybrid) which comprises a catalytically (enzymatically) active amino acid sequence of a non-cellulolytic enzyme linked to an amino acid sequence comprising a cellulose-binding domain.

Stains

Soiling or stains which may be removed according to the present invention include those already mentioned above, i.e. soiling or stains originating from, for example, starch, proteins, fats, red wine, fruit [such as blackcurrant, cherry, strawberry or tomato (in particular tomato in ketchup or spaghetti sauce)], vegetables (such as carrot or beetroot), tea, coffee, spices (such as curry or paprika), body fluids, grass, or ink (e.g. from ball-point pens or fountain pens). Other types of soiling or stains which are appropriate targets for removal or bleaching in accordance with the invention include sebum, soil (i.e. earth), clay, oil and paint.

Cellulosic fabric

The term "cellulosic fabric" is intended to indicate any type of fabric, in particular woven fabric, prepared from a cellulose-containing material, such as cotton, or from a cellulose-

derived material (prepared, e.g., from wood pulp or from cotton).

In the present context, the term "fabric" is intended to include garments and other types of processed fabrics, and is used interchangeably with the term "textile".

Examples of cellulosic fabric manufactured from naturally occurring cellulosic fibre are cotton, ramie, jute and flax (linen) fabrics. Examples of cellulosic fabrics made from man-made cellulosic fibre are viscose (rayon) and lyocell (e.g. Tencel™) fabric; also of relevance in the context of the invention are all blends of cellulosic fibres (such as viscose, lyocell, cotton, ramie, jute or flax) with other fibres, e.g. with animal hair fibres such as wool, alpaca or camel hair, or with polymer fibres such as polyester, polyacrylic, polyamide or polyacetate fibres.

Specific examples of blended cellulosic fabric are viscose/-cotton blends, lyocell/cotton blends (e.g. Tencel™/cotton blends), viscose/wool blends, lyocell/wool blends, cotton/wool blends, cotton/polyester blends, viscose/cotton/polyester blends, wool/cotton/polyester blends, and flax/cotton blends.

Cellulose-binding domains

Although a number of types of carbohydrate-binding domains have been described in the patent and scientific literature, the majority thereof - many of which derive from cellulolytic enzymes (cellulases) - are commonly referred to as "cellulose-binding domains"; a typical cellulose-binding domain (CBD) will thus be one which occurs in a cellulase and which binds preferentially to cellulose and/or to poly- or oligosaccharide fragments thereof.

Cellulose-binding (and other carbohydrate-binding) domains are polypeptide amino acid sequences which occur as integral parts of large polypeptides or proteins consisting of two or more polypeptide amino acid sequence regions, especially in hydrolytic enzymes (hydrolases) which typically comprise a catalytic domain containing the active site for substrate hydrolysis and a carbohydrate-binding domain for binding to the carbohydrate substrate in question. Such enzymes can comprise more than one catalytic domain and one, two or three carbohydrate-binding domains, and they may further comprise one or more polypeptide amino acid sequence regions linking the carbohydrate-binding domain(s) with the catalytic domain(s), a region of the latter type usually being denoted a "linker".

Examples of hydrolytic enzymes comprising a cellulose-binding domain are cellulases, xylanases, mannanases, arabinofuranosidases, acetylesterases and chitinases. "Cellulose-binding domains" have also been found in algae, e.g. in the red alga *Porphyra purpurea* in the form of a non-hydrolytic polysaccharide-binding protein [see P. Tomme et al., Cellulose-Binding Domains - Classification and Properties in Enzymatic Degradation of Insoluble Carbohydrates, John N. Saddler and Michael H. Penner (Eds.), ACS Symposium Series, No. 618 (1996)]. However, most of the known CBDs [which are classified and referred to by P. Tomme et al. (*op cit.*) as "cellulose-binding domains"] derive from cellulases and xylanases.

In the present context, the term "cellulose-binding domain" is intended to be understood in the same manner as in the latter reference (P. Tomme et al., *op. cit*). The P. Tomme et al. reference classifies more than 120 "cellulose-binding domains" into 10 families (I-X) which may have different functions or roles in connection with the mechanism of

substrate binding. However, it is to be anticipated that new family representatives and additional families will appear in the future, and in connection with the present invention a representative of one such new CBD family has in fact been identified (see Example 2 herein).

In proteins/polypeptides in which CBDs occur (e.g. enzymes, typically hydrolytic enzymes such as cellulases), a CBD may be located at the N or C terminus or at an internal position.

That part of a polypeptide or protein (e.g. hydrolytic enzyme) which constitutes a CBD *per se* typically consists of more than about 30 and less than about 250 amino acid residues. For example: those CBDs listed and classified in Family I in accordance with P. Tomme et al. (*op. cit.*) consist of 33-37 amino acid residues, those listed and classified in Family IIa consist of 95-108 amino acid residues, those listed and classified in Family VI consist of 85-92 amino acid residues, whilst one CBD (derived from a cellulase from *Clostridium thermocellum*) listed and classified in Family VII consists of 240 amino acid residues. Accordingly, the molecular weight of an amino acid sequence constituting a CBD *per se* will typically be in the range of from about 4kD to about 40kD, and usually below about 35kD.

Enzyme hybrids

Enzyme classification numbers (EC numbers) referred to in the present specification with claims are in accordance with the Recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, Academic Press Inc., 1992.

A modified enzyme (enzyme hybrid) for use in accordance with the invention comprises a catalytically active (enzymatically

active) amino acid sequence (in general a polypeptide amino acid sequence) of a non-cellulolytic enzyme (i.e. a catalytically active amino acid sequence of an enzyme other than a cellulase) useful in relation to the cleaning of fabric or textile (typically the removal or bleaching of soiling or stains from fabrics or textiles in washing processes), in particular of an enzyme selected from the group consisting of amylases (e.g. α-amylases, EC 3.2.1.1), proteases (i.e. peptidases, EC 3.4), lipases (e.g. triacylglycerol lipases, EC 3.1.1.3) and oxidoreductases (e.g. peroxidases, EC 1.11.1, such as those classified under EC 1.11.1.7; or phenol-oxidizing oxidases, such as laccases, EC 1.10.3.2, or other enzymes classified under EC 1.10.3), fused (linked) to an amino acid sequence comprising a cellulose-binding domain. The catalytically active amino acid sequence in question may comprise or consist of the whole of - or substantially the whole of - the full amino acid sequence of the mature enzyme in question, or it may consist of a portion of the full sequence which retains substantially the same catalytic (enzymatic) properties as the full sequence.

Modified enzymes (enzyme hybrids) of the type in question, as well as detailed descriptions of the preparation and purification thereof, are known in the art [see, e.g., WO 90/00609, WO 94/24158 and WO 95/16782, as well as Greenwood et al., Biotechnology and Bioengineering 44 (1994) pp. 1295-1305]. They may, e.g., be prepared by transforming into a host cell a DNA construct comprising at least a fragment of DNA encoding the cellulose-binding domain ligated, with or without a linker, to a DNA sequence encoding the enzyme of interest, and growing the transformed host cell to express the fused gene. One relevant, but non-limiting, type of recombinant product (enzyme hybrid) obtainable in this manner - often referred to in the art as a "fusion protein" - may be described by one of the following general formulae:

A-CBD-MR-X-B

A-X-MR-CBD-B

In the latter formulae, CBD is an amino acid sequence comprising at least the cellulose-binding domain (CBD) *per se*.

MR (the middle region; a linker) may be a bond, or a linking group comprising from 1 to about 100 amino acid residues, in particular of from 2 to 40 amino acid residues, e.g. from 2 to 15 amino acid residues. MR may, in principle, alternatively be a non-amino-acid linker.

X is an amino acid sequence comprising the above-mentioned, catalytically (enzymatically) active sequence of amino acid residues of a polypeptide encoded by a DNA sequence encoding the non-cellulolytic enzyme of interest.

The moieties A and B are independently optional. When present, a moiety A or B constitutes a terminal extension of a CBD or X moiety, and normally comprises one or more amino acid residues.

It will thus, *inter alia*, be apparent from the above that a CBD in an enzyme hybrid of the type in question may be positioned C-terminally, N-terminally or internally in the enzyme hybrid. Correspondingly, an X moiety in an enzyme hybrid of the type in question may be positioned N-terminally, C-terminally or internally in the enzyme hybrid.

Enzyme hybrids of interest in the context of the invention include enzyme hybrids which comprise more than one CBD, e.g. such that two or more CBDs are linked directly to each other, or are separated from one another by means of spacer or linker

sequences (consisting typically of a sequence of amino acid residues of appropriate length). Two CBDs in an enzyme hybrid of the type in question may, for example, also be separated from one another by means of an -MR-X- moiety as defined above.

A very important issue in the construction of enzyme hybrids of the type in question is the stability towards proteolytic degradation. Two- and multi-domain proteins are particularly susceptible towards proteolytic cleavage of linker regions connecting the domains. Proteases causing such cleavage may, for example, be subtilisins, which are known to often exhibit broad substrate specificities [see, e.g.: Grøn et al., Biochemistry 31 (1992), pp. 6011-6018; Teplyakov et al., Protein Engineering 5 (1992), pp. 413-420].

Glycosylation of linker residues in eukaryotes is one of Nature's ways of preventing proteolytic degradation. Another is to employ amino acids which are less favoured by the surrounding proteases. The length of the linker also plays a role in relation to accessibility by proteases. Which "solution" is optimal depends on the environment in which the enzyme hybrid is to function.

When constructing new enzyme hybrid molecules, linker stability
thus becomes an issue of great importance. The various linkers described in examples presented herein (*vide infra*) in the context of the present invention are intended to take account of this issue.

Cellulases (cellulase genes) useful for preparation of CBDs

Techniques suitable for isolating a cellulase gene are well known in the art. In the present context, the terms "cellulase" and "cellulolytic enzyme" refer to an enzyme which

catalyses the degradation of cellulose to glucose, cellobiose, triose and/or other cello-oligosaccharides.

Preferred cellulases (i.e. cellulases comprising preferred CBDs) in the present context are microbial cellulases, particularly bacterial or fungal cellulases. Endoglucanases, notably endo-1,4-β-glucanases (EC 3.2.1.4), particularly monocomponent (recombinant) endo-1,4-β-glucanases, are a preferred class of cellulases,.

Useful examples of bacterial cellulases are cellulases derived from or producible by bacteria from the group consisting of *Pseudomonas*, *Bacillus*, *Cellulomonas*, *Clostridium*, *Microspora*, *Thermotoga*, *Caldocellum* and Actinomycets such as *Streptomyces*, *Termomonospora* and *Acidothemus*, in particular from the group consisting of *Pseudomonas cellulolyticus*, *Bacillus lautus*, *Cellulomonas fimi*, *Clostridium thermocellum*, *Microspora bispora*, *Termomonospora fusca*, *Termomonospora cellulolyticum* and *Acidothemus cellulolyticus*.

The cellulase may be an acid, a neutral or an alkaline cellulase, i.e. exhibiting maximum cellulolytic activity in the acid, neutral or alkaline range, respectively.

A useful cellulase is an acid cellulase, preferably a fungal acid cellulase, which is derived from or producible by fungi from the group of genera consisting of *Trichoderma*, *Myrothecium*, *Aspergillus*, *Phanaerochaete*, *Neurospora*, *Neocallimastix* and *Botrytis*.

A preferred useful acid cellulase is one derived from or producible by fungi from the group of species consisting of *Trichoderma viride*, *Trichoderma reesei*, *Trichoderma longibrachiatum*, *Myrothecium verrucaria*, *Aspergillus niger*, *Aspergil-*

lus oryzae, *Phanaerochaete chrysosporium*, *Neurospora crassa*, *Neocallimastix partriciarum* and *Botrytis cinerea*.

Another useful cellulase is a neutral or alkaline cellulase, preferably a fungal neutral or alkaline cellulase, which is derived from or producible by fungi from the group of genera consisting of *Aspergillus*, *Penicillium*, *Myceliophthora*, *Humicola*, *Irpex*, *Fusarium*, *Stachybotrys*, *Scopulariopsis*, *Chaetomium*, *Mycogone*, *Verticillium*, *Myrothecium*, *Papulospora*, *Gliocladium*, *Cephalosporium* and *Acremonium*.

A preferred alkaline cellulase is one derived from or producible by fungi from the group of species consisting of *Humicola insolens*, *Fusarium oxysporum*, *Myceliopthora thermophila*, *Penicillium janthinellum* and *Cephalosporium* sp., preferably from the group of species consisting of *Humicola insolens* DSM 1800, *Fusarium oxysporum* DSM 2672, *Myceliopthora thermophila* CBS 117.65, and *Cephalosporium* sp. RYM-202.

A preferred cellulase is an alkaline endoglucanase which is immunologically reactive with an antibody raised against a highly purified ~43kD endoglucanase derived from *Humicola insolens* DSM 1800, or which is a derivative of the latter ~43kD endoglucanase and exhibits cellulase activity.

Other examples of useful cellulases are variants of parent cellulases of fungal or bacterial origin, e.g. variants of a parent cellulase derivable from a strain of a species within one of the fungal genera *Humicola*, *Trichoderma* or *Fusarium*.

Isolation of a cellulose-binding domain

In order to isolate a cellulose-binding domain of, e.g., a cellulase, several genetic engineering approaches may be used. One method uses restriction enzymes to remove a portion of the

gene and then to fuse the remaining gene-vector fragment in frame to obtain a mutated gene that encodes a protein truncated for a particular gene fragment. Another method involves the use of exonucleases such as *Ba*l31 to systematically delete nucleotides either externally from the 5' and the 3' ends of the DNA or internally from a restricted gap within the gene. These gene-deletion methods result in a mutated gene encoding a shortened gene molecule whose expression product may then be evaluated for substrate-binding (e.g. cellulose-binding) ability. Appropriate substrates for evaluating the binding ability include cellulosic materials such as Avicel™ and cotton fibres. Other methods include the use of a selective or specific protease capable of cleaving a CBD, e.g. a terminal CBD, from the remainder of the polypeptide chain of the protein in question

As already indicated (*vide supra*), once a nucleotide sequence encoding the substrate-binding (carbohydrate-binding) region has been identified, either as cDNA or chromosomal DNA, it may then be manipulated in a variety of ways to fuse it to a DNA sequence encoding the enzyme or enzymatically active amino acid sequence of interest. The DNA fragment encoding the carbohydrate-binding amino acid sequence, and the DNA encoding the enzyme or enzymatically active amino acid sequence of interest are then ligated with or without a linker. The resulting ligated DNA may then be manipulated in a variety of ways to achieve expression. Preferred microbial expression hosts include certain *Aspergillus* species (e.g. *A. niger* or *A. oryzae*), *Bacillus* species, and organisms such as *Escherichia coli* or *Saccharomyces cerevisiae*.

Amylolytic enzymes

Amylases (e.g. α- or β-amylases) which are appropriate as the basis for enzyme hybrids of the types employed in the context

of the present invention include those of bacterial or fungal origin. Chemically or genetically modified mutants of such amylases are included in this connection. Relevant α-amylases include, for example, α-amylases obtainable from *Bacillus* species, in particular a special strain of *B. licheniformis*, described in more detail in GB 1296839. Relevant commercially available amylases include Duramyl™, Termamyl™, Fungamyl™ and BAN™ (all available from Novo Nordisk A/S, Bagsvaerd, Denmark), and Rapidase™ and Maxamyl™ P (available from Gist-Brocades, Holland).

Other useful amylolytic enzymes are CGTases (cyclodextrin glucanotransferases, EC 2.4.1.19), e.g. those obtainable from species of *Bacillus*, *Thermoanaerobactor* or *Thermoanaerobacterium*.

Proteolytic enzymes

Proteases (peptidases) which are appropriate as the basis for enzyme hybrids of the types employed in the context of the present invention include those of animal, vegetable or microbial origin. Proteases of microbial origin are preferred. Chemically or genetically modified mutants of such proteases are included in this connection. The protease may be a serine protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the Fusarium protease described in WO 89/06270.

Relevant commercially available protease enzymes include

Alcalase™, Savinase™, Primase, Durazym™ and Esperase™ (all available from Novo Nordisk A/S, Bagsvaerd, Denmark), Maxatase™, Maxacal™, Maxapem™ and Properase™ (available from Gist-Brocades, Holland), Purafect™ and Purafect™ OXP (available from Genencor International), and Opticlean™ and Optimase™ (available from by Solvay Enzymes).

Lipolytic enzymes

Lipolytic enzymes (lipases) which are appropriate as the basis for enzyme hybrids of the types employed in the context of the present invention include those of bacterial or fungal origin. Chemically or genetically modified mutants of such lipases are included in this connection.

Examples of useful lipases include a *Humicola lanuginosa* lipase, e.g. as described in EP 258 068 and EP 305 216; a *Rhizomucor miehei* lipase, e.g. as described in EP 238 023; a *Candida* lipase, such as a *C. antarctica* lipase, e.g. the *C. antarctica* lipase A or B described in EP 214 761; a *Pseudomonas lipase*, such as one of those described in EP 721 981 (e.g. a lipase obtainable from a *Pseudomonas* sp. SD705 strain having deposit accession number FERM BP-4772), in PCT/JP96/00426, in PCT/JP96/00454 (e.g. a *P. solanacearum* lipase), in EP 571 982 or in WO 95/14783 (e.g. a *P. mendocina* lipase), a *P. alcaligenes* or *P. pseudoalcaligenes* lipase, e.g. as described in EP 218 272, a *P. cepacia* lipase, e.g. as described in EP 331 376, a *P. stutzeri* lipase, e.g. as disclosed in GB 1,372,034, or a *P. fluorescens* lipase; a *Bacillus* lipase, e.g. a *B. subtilis* lipase [Dartois et al., Biochemica et Biophysica Acta 1131 (1993) pp. 253-260], a *B. stearothermophilus* lipase (JP 64/744992) and a *B. pumilus* lipase (WO 91/16422).

Furthermore, a number of cloned lipases may be useful, including the *Penicillium camembertii* lipase described by Yamaguchi et al. in Gene 103 (1991), pp. 61-67, the *Geotricum candidum* lipase [Y. Schimada et al., J. Biochem. 106 (1989), pp. 383-388], and various *Rhizopus* lipases such as an *R. delemar* lipase [M.J. Hass et al., Gene 109 (1991) pp. 117-113], an *R. niveus* lipase [Kugimiya et al., Biosci. Biotech. Biochem. 56 (1992), pp. 716-719] and a *R. oryzae* lipase.

Other potentially useful types of lipolytic enzymes include cutinases, e.g. a cutinase derived from *Pseudomonas mendocina* as described in WO 88/09367, or a cutinase derived from *Fusarium solani* f. pisi (described, e.g., in WO 90/09446).

Suitable commercially available lipases include Lipolase™ and Lipolase Ultra™ (available from Novo Nordisk A/S), M1 Lipase™, Lumafast™ and Lipomax™ (available from Gist-Brocades) and Lipase P "Amano" (available from Amano Pharmaceutical Co. Ltd.).

Oxidoreductases

Oxidoreductases which are appropriate as the basis for enzyme hybrids of the types employed in the context of the present invention include peroxidases (EC 1.11.1) and oxidases, such as laccases (EC 1.10.3.2) and certain related enzymes.

Peroxidases

Peroxidases (EC 1.11.1) are enzymes acting on a peroxide (e.g. hydrogen peroxide) as acceptor. Very suitable peroxidases are those classified under EC 1.11.1.7, or any fragment derived therefrom, exhibiting peroxidase activity. Synthetic or semisynthetic derivatives thereof (e.g. with porphyrin ring

systems, or microperoxidases, cf., for example, US 4,077,768, EP 537 381, WO 91/05858 and WO 92/16634) may also be of value in the context of the invention.

Very suitable peroxidases are peroxidases obtainable from plants (e.g. horseradish peroxidase or soy bean peroxidase) or from microorganisms, such as fungi or bacteria. In this respect, some preferred fungi include strains belonging to the subdivision Deuteromycotina, class Hyphomycetes, e.g. *Fusarium*, *Humicola*, *Tricoderma*, *Myrothecium*, *Verticillum*, *Arthromyces*, *Caldariomyces*, *Ulocladium*, *Embellisia*, *Cladosporium* or *Dreschlera*, in particular *Fusarium oxysporum* (DSM 2672), *Humicola insolens*, *Trichoderma resii*, *Myrothecium verrucana* (IFO 6113), *Verticillum alboatrum*, *Verticillum dahlie*, *Arthromyces ramosus* (FERM P-7754), *Caldariomyces fumago*, *Ulocladium chartarum*, *Embellisia alli* or *Dreschlera halodes*.

Other preferred fungi include strains belonging to the subdivision Basidiomycotina, class Basidiomycetes, e.g. *Coprinus*, *Phanerochaete*, *Coriolus* or *Trametes*, in particular *Coprinus cinereus* f. *microsporus* (IFO 8371), *Coprinus macrorhizus*, *Phanerochaete chrysosporium* (e.g. NA-12) or *Trametes versicolor* (e.g. PR4 28-A).

Further preferred fungi include strains belonging to the subdivision Zygomycotina, class Mycoraceae, e.g. *Rhizopus* or *Mucor*, in particular *Mucor hiemalis*.

Some preferred bacteria include strains of the order Actinomycetales, e.g. *Streptomyces spheroides* (ATTC 23965), *Streptomyces thermoviolaceus* (IFO 12382) or *Streptoverticillum verticillium* ssp. *verticillium*.

Other preferred bacteria include *Bacillus pumilus* (ATCC 12905), *Bacillus stearothermophilus*, *Rhodobacter sphaeroides*, *Rhodomonas palustri*, *Streptococcus lactis*, *Pseudomonas purrocinia* (ATCC 15958) or *Pseudomonas fluorescens* (NRRL B-11).

Further preferred bacteria include strains belonging to *Myxococcus*, e.g. *M. virescens*.

Other potential sources of useful particular peroxidases are listed in B.C. Saunders et al., *Peroxidase*, London 1964, pp. 41-43.

The peroxidase may furthermore be one which is producible by a method comprising cultivating a host cell - transformed with a recombinant DNA vector which carries a DNA sequence encoding said peroxidase as well as DNA sequences encoding functions permitting the expression of the DNA sequence encoding the peroxidase - in a culture medium under conditions permitting the expression of the peroxidase, and recovering the peroxidase from the culture.

A suitable recombinantly produced peroxidase is a peroxidase derived from a *Coprinus* sp., in particular *C. macrorhizus* or *C. cinereus* according to WO 92/16634, or a variant thereof, e.g. a variant as described in WO 94/12621.

Oxidases and related enzymes

preferred oxidases in the context of the present invention are oxidases classified under EC 1.10.3, which are oxidases employing molecular oxygen as acceptor (i.e. enzymes catalyzing oxidation reactions in which molecular oxygen functions as oxidizing agent).

As indicated above, laccases (EC 1.10.3.2) are very suitable oxidases in the context of the invention. Examples of other useful oxidases in the context of the invention include the catechol oxidases (EC 1.10.3.1) and bilirubin oxidases (EC 1.3.3.5). Further useful, related enzymes include monophenol monooxygenases (EC 1.14.18.1).

Laccases are obtainable from a variety of plant and microbial sources, notably from bacteria and fungi (including filamentous fungi and yeasts), and suitable examples of laccases are to found among those obtainable from fungi, including laccases obtainable from strains of *Aspergillus*, *Neurospora* (e.g. *N. crassa*), *Podospora*, *Botrytis*, *Collybia*, *Fomes*, *Lentinus*, *Pleurotus*, *Trametes* (e.g. *T. villosa* or *T. versicolor* [some species/strains of *Trametes* being known by various names and/or having previously been classified within other genera; e.g. *Trametes villosa* = *T. pinsitus* = *Polyporus pinsitis* (also known as *P. pinsitus* or *P. villosus*) = *Coriolus pinsitus*], *Polyporus*, *Rhizoctonia* (e.g. *R. solani*), *Coprinus* (e.g. *C. plicatilis* or *C. cinereus*), *Psatyrella*, *Myceliophthora* (e.g. *M. thermophila*), *Schytalidium*, *Phlebia* (e.g. *P. radita*; see WO 92/01046), *Coriolus* (e.g. *C.hirsutus*; see JP 2-238885), *Pyricularia* or *Rigidoporus*.

Preferred laccases in the context of the invention include laccase obtainable from species/strains of *Trametes* (e.g. *T. villosa*), *Myceliophthora* (e.g. *M. thermophila*), *Schytalidium* or *Polyporus*.

Other enzymes

Further classes of enzymes which are appropriate as the basis for enzyme hybrids of the types employed in the context of the

present invention include pectinases (polygalacturonases; EC 3.2.1.15).

Plasmids

Preparation of plasmids capable of expressing fusion proteins having the amino acid sequences derived from fragments of more than one polypeptide is well known in the art (see, for example, WO 90/00609 and WO 95/16782). The expression cassette may be included within a replication system for episomal maintenance in an appropriate cellular host or may be provided without a replication system, where it may become integrated into the host genome. The DNA may be introduced into the host in accordance with known techniques such as transformation, microinjection or the like.

Once the fused gene has been introduced into the appropriate host, the host may be grown to express the fused gene. Normally it is desirable additionally to add a signal sequence which provides for secretion of the fused gene. Typical examples of useful fused genes are:

Signal sequence -- (pro-peptide) -- carbohydrate-binding domain -- linker -- enzyme sequence of interest, or

Signal sequence -- (pro-peptide) -- enzyme sequence of interest -- linker -- carbohydrate-binding domain,

in which the pro-peptide sequence normally contains 5-100, e.g. 5-25, amino acid residues.

The recombinant product may be glycosylated or non-glycosylated.

Detergent compositions

Surfactant system

The detergent compositions according to the present invention comprise a surfactant system, wherein the surfactant can be selected from nonionic and/or anionic and/or cationic and/or ampholytic and/or zwitterionic and/or semi-polar surfactants.

The surfactant is typically present at a level from 0.1% to 60% by weight. The surfactant is preferably formulated to be compatible with enzyme hybrid and enzyme components present in the composition. In liquid or gel compositions the surfactant is most preferably formulated in such a way that it promotes, or at least does not degrade, the stability of any enzyme hybrid or enzyme in these compositions.

Suitable systems for use according to the present invention comprise as a surfactant one or more of the nonionic and/or anionic surfactants described herein.

Polyethylene, polypropylene, and polybutylene oxide condensates of alkyl phenols are suitable for use as the nonionic surfactant of the surfactant systems of the present invention, with the polyethylene oxide condensates being preferred. These compounds include the condensation products of alkyl phenols having an alkyl group containing from about 6 to about 14 carbon atoms, preferably from about 8 to about 14 carbon atoms, in either a straight chain or branched-chain configuration with the alkylene oxide. In a preferred embodiment, the ethylene oxide is present in an amount equal to from about 2 to about 25 moles, more preferably from about 3 to about 15 moles, of ethylene oxide per mole of alkyl phenol. Commercially available nonionic surfactants of this type include Igepal™ CO-630, marketed by the GAF Corporation; and Triton™ X-45, X-114, X-100 and X-102, all marketed by the Rohm & Haas Company. These surfactants are commonly referred to as alkylphenol alkoxylates (e.g., alkyl phenol

ethoxylates).

The condensation products of primary and secondary aliphatic alcohols with about 1 to about 25 moles of ethylene oxide are suitable for use as the nonionic surfactant of the nonionic surfactant systems of the present invention. The alkyl chain of the aliphatic alcohol can either be straight or branched, primary or secondary, and generally contains from about 8 to about 22 carbon atoms. Preferred are the condensation products of alcohols having an alkyl group containing from about 8 to about 20 carbon atoms, more preferably from about 10 to about 18 carbon atoms, with from about 2 to about 10 moles of ethylene oxide per mole of alcohol. About 2 to about 7 moles of ethylene oxide and most preferably from 2 to 5 moles of ethylene oxide per mole of alcohol are present in said condensation products. Examples of commercially available nonionic surfactants of this type include Tergitol™ 15-S-9 (The condensation product of C_{11}-C_{15} linear alcohol with 9 moles ethylene oxide), Tergitol™ 24-L-6 NMW (the condensation product of C_{12}-C_{14} primary alcohol with 6 moles ethylene oxide with a narrow molecular weight distribution), both marketed by Union Carbide Corporation; Neodol™ 45-9 (the condensation product of C_{14}-C_{15} linear alcohol with 9 moles of ethylene oxide), Neodol™ 23-3 (the condensation product of C_{12}-C_{13} linear alcohol with 3.0 moles of ethylene oxide), Neodol™ 45-7 (the condensation product of C_{14}-C_{15} linear alcohol with 7 moles of ethylene oxide), Neodol™ 45-5 (the condensation product of C_{14}-C_{15} linear alcohol with 5 moles of ethylene oxide) marketed by Shell Chemical Company, Kyro™ EOB (the condensation product of C_{13}-C_{15} alcohol with 9 moles ethylene oxide), marketed by The Procter & Gamble Company, and Genapol LA 050 (the condensation product of C_{12}-C_{14} alcohol with 5 moles of ethylene oxide) marketed by Hoechst. Preferred range of HLB in these products is from 8-11 and most preferred from 8-10.

Also useful as the nonionic surfactant of the surfactant systems of the present invention are alkylpolysaccharides disclosed in US 4,565,647, having a hydrophobic group containing from about 6 to about 30 carbon atoms, preferably from about 10 to about 16 carbon atoms and a polysaccharide, e.g. a polyglycoside, hydrophilic group containing from about 1.3 to about 10, preferably from about 1.3 to about 3, most preferably from about 1.3 to about 2.7 saccharide units. Any reducing saccharide containing 5 or 6 carbon atoms can be used, e.g., glucose, galactose and galactosyl moieties can be substituted for the glucosyl moieties (optionally the hydrophobic group is attached at the 2-, 3-, 4-, etc. positions thus giving a glucose or galactose as opposed to a glucoside or galactoside). The intersaccharide bonds can be, e.g., between the one position of the additional saccharide units and the 2-, 3-, 4-, and/or 6- positions on the preceding saccharide units.

The preferred alkylpolyglycosides have the formula

$$R^2O(C_nH_{2n}O)_t(\text{glycosyl})_x$$

wherein R^2 is selected from the group consisting of alkyl, alkylphenyl, hydroxyalkyl, hydroxyalkylphenyl, and mixtures thereof in which the alkyl groups contain from about 10 to about 18, preferably from about 12 to about 14, carbon atoms; n is 2 or 3, preferably 2; t is from 0 to about 10, preferably 0; and x is from about 1.3 to about 10, preferably from about 1.3 to about 3, most preferably from about 1.3 to about 2.7. The glycosyl is preferably derived from glucose. To prepare these compounds, the alcohol or alkylpolyethoxy alcohol is formed first and then reacted with glucose, or a source of glucose, to form the glucoside (attachment at the 1-position). The additional glycosyl units can then be

attached between their 1-position and the preceding glycosyl units 2-, 3-, 4-, and/or 6-position, preferably predominantly the 2-position.

The condensation products of ethylene oxide with a hydrophobic base formed by the condensation of propylene oxide with propylene glycol are also suitable for use as the additional nonionic surfactant systems of the present invention. The hydrophobic portion of these compounds will preferably have a molecular weight from about 1500 to about 1800 and will exhibit water insolubility. The addition of polyoxyethylene moieties to this hydrophobic portion tends to increase the water solubility of the molecule as a whole, and the liquid character of the product is retained up to the point where the polyoxyethylene content is about 50% of the total weight of the condensation product, which corresponds to condensation with up to about 40 moles of ethylene oxide. Examples of compounds of this type include certain of the commercially available Pluronic™ surfactants, marketed by BASF.

Also suitable for use as the nonionic surfactant of the nonionic surfactant system of the present invention, are the condensation products of ethylene oxide with the product resulting from the reaction of propylene oxide and ethylenediamine. The hydrophobic moiety of these products consists of the reaction product of ethylenediamine and excess propylene oxide, and generally has a molecular weight of from about 2500 to about 3000. This hydrophobic moiety is condensed with ethylene oxide to the extent that the condensation product contains from about 40% to about 80% by weight of polyoxyethylene and has a molecular weight of from about 5,000 to about 11,000. Examples of this type of nonionic surfactant include certain of the commercially available Tetronic™ compounds, marketed by BASF.

Preferred for use as the nonionic surfactant of the surfactant systems of the present invention are polyethylene oxide condensates of alkyl phenols, condensation products of primary and secondary aliphatic alcohols with from about 1 to about 25 moles of ethyleneoxide, alkylpolysaccharides, and mixtures hereof. Most preferred are C_8-C_{14} alkyl phenol ethoxylates having from 3 to 15 ethoxy groups and C_8-C_{18} alcohol ethoxylates (preferably C_{10} avg.) having from 2 to 10 ethoxy groups, and mixtures thereof.

Highly preferred nonionic surfactants are polyhydroxy fatty acid amide surfactants of the formula

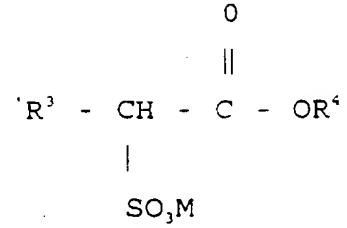
$$R^2 - C(=O) - N(R^1) - Z,$$

wherein R^1 is H, or R^1 is C_{1-4} hydrocarbyl, 2-hydroxyethyl, 2-hydroxypropyl or a mixture thereof, R^2 is C_{5-31} hydrocarbyl, and Z is a polyhydroxyhydrocarbyl having a linear hydrocarbyl chain with at least 3 hydroxyls directly connected to the chain, or an alkoxylated derivative thereof. Preferably, R^1 is methyl, R^2 is straight C_{11-15} alkyl or C_{16-18} alkyl or alkenyl chain such as coconut alkyl or mixtures thereof, and Z is derived from a reducing sugar such as glucose, fructose, maltose or lactose, in a reductive amination reaction.

Highly preferred anionic surfactants include alkyl alkoxylated sulfate surfactants. Examples hereof are water soluble salts or acids of the formula $RO(A)_mSO3M$ wherein R is an unsubstituted C_{10}-C_{24} alkyl or hydroxyalkyl group having a C_{10}-C_{24} alkyl component, preferably a C_{12}-C_{20} alkyl or hydroxyalkyl, more preferably C_{12}-C_{18} alkyl or hydroxyalkyl, A is an ethoxy or propoxy unit, m is greater than zero, typically

between about 0.5 and about 6, more preferably between about 0.5 and about 3, and M is H or a cation which can be, for example, a metal cation (e.g., sodium, potassium, lithium, calcium, magnesium, etc.), ammonium or substituted-ammonium cation. Alkyl ethoxylated sulfates as well as alkyl propoxylated sulfates are contemplated herein. Specific examples of substituted ammonium cations include methyl-, dimethyl, trimethyl-ammonium cations and quaternary ammonium cations such as tetramethyl-ammonium and dimethyl piperdinium cations and those derived from alkylamines such as ethylamine, diethylamine, triethylamine, mixtures thereof, and the like. Exemplary surfactants are C_{12}-C_{18} alkyl polyethoxylate (1.0) sulfate (C_{12}-C_{18}E(1.0)M), C_{12}-C_{18} alkyl polyethoxylate (2.25) sulfate (C_{12}-C_{18}(2.25)M, and C_{12}-C_{18} alkyl polyethoxylate (3.0) sulfate (C_{12}-C_{18}E(3.0)M), and C_{12}-C_{18} alkyl polyethoxylate (4.0) sulfate (C_{12}-C_{18}E(4.0)M), wherein M is conveniently selected from sodium and potassium.
Suitable anionic surfactants to be used are alkyl ester sulfonate surfactants including linear esters of C_8-C_{20} carboxylic acids (i.e., fatty acids) which are sulfonated with gaseous SO_3 according to "The Journal of the American Oil Chemists Society", 52 (1975), pp. 323-329. Suitable starting materials would include natural fatty substances as derived from tallow, palm oil, etc.

The preferred alkyl ester sulfonate surfactant, especially for laundry applications, comprise alkyl ester sulfonate surfactants of the structural formula:



wherein R^3 is a C_8-C_{20} hydrocarbyl, preferably an alkyl, or combination thereof, R^4 is a C_1-C_6 hydrocarbyl, preferably an alkyl, or combination thereof, and M is a cation which forms a water soluble salt with the alkyl ester sulfonate. Suitable salt-forming cations include metals such as sodium, potassium, and lithium, and substituted or unsubstituted ammonium cations, such as monoethanolamine, diethonolamine, and triethanolamine. Preferably, R^3 is C_{10}-C_{16} alkyl, and R^4 is methyl, ethyl or isopropyl. Especially preferred are the methyl ester sulfonates wherein R^3 is C_{10}-C_{16} alkyl.

Other suitable anionic surfactants include the alkyl sulfate surfactants which are water soluble salts or acids of the formula $ROSO_3M$ wherein R preferably is a C_{10}-C_{24} hydrocarbyl, preferably an alkyl or hydroxyalkyl having a C_{10}-C_{20} alkyl component, more preferably a C_{12}-C_{18} alkyl or hydroxyalkyl, and M is H or a cation, e.g., an alkali metal cation (e.g. sodium, potassium, lithium), or ammonium or substituted ammonium (e.g. methyl-, dimethyl-, and trimethyl ammonium cations and quaternary ammonium cations such as tetramethyl-ammonium and dimethyl piperdinium cations and quaternary ammonium cations derived from alkylamines such as ethylamine, diethylamine, triethylamine, and mixtures thereof, and the like). Typically, alkyl chains of C_{12}-C_{16} are preferred for lower wash temperatures (e.g. below about 50°C) and C_{16}-C_{18} alkyl chains are preferred for higher wash temperatures (e.g. above about 50°C).

Other anionic surfactants useful for detersive purposes can also be included in the laundry detergent compositions of the present invention. Theses can include salts (including, for example, sodium, potassium, ammonium, and substituted ammonium salts such as mono- di- and triethanolamine salts) of soap, C_8-C_{22} primary or secondary alkanesulfonates, C_8-C_{24}

olefinsulfonates, sulfonated polycarboxylic acids prepared by sulfonation of the pyrolyzed product of alkaline earth metal citrates, e.g., as described in British patent specification No. 1,082,179, C_8-C_{24} alkylpolyglycolethersulfates (containing up to 10 moles of ethylene oxide); alkyl glycerol sulfonates, fatty acyl glycerol sulfonates, fatty oleyl glycerol sulfates, alkyl phenol ethylene oxide ether sulfates, paraffin sulfonates, alkyl phosphates, isethionates such as the acyl isethionates, N-acyl taurates, alkyl succinamates and sulfosuccinates, monoesters of sulfosuccinates (especially saturated and unsaturated C_{12}-C_{18} monoesters) and diesters of sulfosuccinates (especially saturated and unsaturated C_6-C_{12} diesters), acyl sarcosinates, sulfates of alkylpolysaccharides such as the sulfates of alkylpolyglucoside (the nonionic nonsulfated compounds being described below), branched primary alkyl sulfates, and alkyl polyethoxy carboxylates such as those of the formula $RO(CH_2CH_2O)_k$-CH_2COO-M+ wherein R is a C_8-C_{22} alkyl, k is an integer from 1 to 10, and M is a soluble salt forming cation. Resin acids and hydrogenated resin acids are also suitable, such as rosin, hydrogenated rosin, and resin acids and hydrogenated resin acids present in or derived from tall oil.

Alkylbenzene sulfonates are highly preferred. Especially preferred are linear (straight-chain) alkyl benzene sulfonates (LAS) wherein the alkyl group preferably contains from 10 to 18 carbon atoms.

Further examples are described in "Surface Active Agents and Detergents" (Vol. I and II by Schwartz, Perrry and Berch). A variety of such surfactants are also generally disclosed in US 3,929,678, (Column 23, line 58 through Column 29, line 23, herein incorporated by reference).

When included therein, the laundry detergent compositions of

the present invention typically comprise from about 1% to about 40%, preferably from about 3% to about 20% by weight of such anionic surfactants.

The laundry detergent compositions of the present invention may also contain cationic, ampholytic, zwitterionic, and semi-polar surfactants, as well as the nonionic and/or anionic surfactants other than those already described herein.

Cationic detersive surfactants suitable for use in the laundry detergent compositions of the present invention are those having one long-chain hydrocarbyl group. Examples of such cationic surfactants include the ammonium surfactants such as alkyltrimethylammonium halogenides, and those surfactants having the formula:

$$[R^2(OR^3)_y][R^4(OR^3)_y]_2R^5N+X-$$

wherein R^2 is an alkyl or alkyl benzyl group having from about 8 to about 18 carbon atoms in the alkyl chain, each R^3 is selected form the group consisting of $-CH_2CH_2-$, $-CH_2CH(CH_3)-$, $-CH_2CH(CH_2OH)-$, $-CH_2CH_2CH_2-$, and mixtures thereof; each R^4 is selected from the group consisting of C_1-C_4 alkyl, C_1-C_4 hydroxyalkyl, benzyl ring structures formed by joining the two R^4 groups, $-CH_2CHOHCHOHCOR^6CHOHCH_2OH$, wherein R^6 is any hexose or hexose polymer having a molecular weight less than about 1000, and hydrogen when y is not 0; R^5 is the same as R^4 or is an alkyl chain,wherein the total number of carbon atoms or R^2 plus R^5 is not more than about 18; each y is from 0 to about 10,and the sum of the y values is from 0 to about 15; and X is any compatible anion.

Highly preferred cationic surfactants are the water soluble quaternary ammonium compounds useful in the present

composition having the formula:

$$R_1R_2R_3R_4N^+X^- \quad (i)$$

wherein R_1 is C_8-C_{16} alkyl, each of R_2, R_3 and R_4 is independently C_1-C_4 alkyl, C_1-C_4 hydroxy alkyl, benzyl, and -$(C_2H_{40})_xH$ where x has a value from 2 to 5, and X is an anion. Not more than one of R_2, R_3 or R_4 should be benzyl.

The preferred alkyl chain length for R_1 is C_{12}-C_{15}, particularly where the alkyl group is a mixture of chain lengths derived from coconut or palm kernel fat or is derived synthetically by olefin build up or OXO alcohols synthesis.

Preferred groups for R_2R_3 and R_4 are methyl and hydroxyethyl groups and the anion X may be selected from halide, methosulphate, acetate and phosphate ions.

Examples of suitable quaternary ammonium compounds of formulae (i) for use herein are:

coconut trimethyl ammonium chloride or bromide;
coconut methyl dihydroxyethyl ammonium chloride or bromide;
decyl triethyl ammonium chloride;
decyl dimethyl hydroxyethyl ammonium chloride or bromide;
C_{12-15} dimethyl hydroxyethyl ammonium chloride or bromide;
coconut dimethyl hydroxyethyl ammonium chloride or bromide;
myristyl trimethyl ammonium methyl sulphate;
lauryl dimethyl benzyl ammonium chloride or bromide;
lauryl dimethyl (ethenoxy)$_4$ ammonium chloride or bromide;
choline esters (compounds of formula (i) wherein R_1 is

$$CH_2\text{-}CH_2\text{-}O\text{-}\overset{\overset{O}{\|}}{C}\text{-}C_{12-14}$$ alkyl and $R_2R_3R_4$ are methyl).

di-alkyl imidazolines [compounds of formula (i)].

Other cationic surfactants useful herein are also described in US 4,228,044 and in EP 000 224.

When included therein, the laundry detergent compositions of the present invention typically comprise from 0.2% to about 25%, preferably from about 1% to about 8% by weight of such cationic surfactants.

Ampholytic surfactants are also suitable for use in the laundry detergent compositions of the present invention. These surfactants can be broadly described as aliphatic derivatives of secondary or tertiary amines, or aliphatic derivatives of heterocyclic secondary and tertiary amines in which the aliphatic radical can be straight- or branched-chain. One of the aliphatic substituents contains at least about 8 carbon atoms, typically from about 8 to about 18 carbon atoms, and at least one contains an anionic water-solubilizing group, e.g. carboxy, sulfonate, sulfate. See US 3,929,678 (column 19, lines 18-35) for examples of ampholytic surfactants.

When included therein, the laundry detergent compositions of the present invention typically comprise from 0.2% to about 15%, preferably from about 1% to about 10% by weight of such ampholytic surfactants.

Zwitterionic surfactants are also suitable for use in laundry detergent compositions. These surfactants can be broadly described as derivatives of secondary and tertiary amines, derivatives of heterocyclic secondary and tertiary amines, or derivatives of quaternary ammonium, quaternary phosphonium or tertiary sulfonium compounds. See US 3,929,678 (column 19,

line 38 through column 22, line 48) for examples of zwitterionic surfactants.

When included therein, the laundry detergent compositions of the present invention typically comprise from 0.2% to about 15%, preferably from about 1% to about 10% by weight of such zwitterionic surfactants.

Semi-polar nonionic surfactants are a special category of nonionic surfactants which include water-soluble amine oxides containing one alkyl moiety of from about 10 to about 18 carbon atoms and 2 moieties selected from the group consisting of alkyl groups and hydroxyalkyl groups containing from about 1 to about 3 carbon atoms; watersoluble phosphine oxides containing one alkyl moiety of from about 10 to about 18 carbon atoms and 2 moieties selected from the group consisting of alkyl groups and hydroxyalkyl groups containing from about 1 to about 3 carbon atoms; and water-soluble sulfoxides containing one alkyl moiety from about 10 to about 18 carbon atoms and a moiety selected from the group consisting of alkyl and hydroxyalkyl moieties of from about 1 to about 3 carbon atoms.

Semi-polar nonionic detergent surfactants include the amine oxide surfactants having the formula:

$$R^3(OR^4)xN(R^5)2 \rightarrow O$$

wherein R^3 is an alkyl, hydroxyalkyl, or alkyl phenyl group or mixtures thereof containing from about 8 to about 22 carbon atoms; R^4 is an alkylene or hydroxyalkylene group containing from about 2 to about 3 carbon atoms or mixtures thereof; x is from 0 to about 3: and each R^5 is an alkyl or

hydroxyalkyl group containing from about 1 to about 3 carbon atoms or a polyethylene oxide group containing from about 1 to about 3 ethylene oxide groups. The R^5 groups can be attached to each other, e.g., through an oxygen or nitrogen atom, to form a ring structure.

These amine oxide surfactants in particular include C_{10}-C_{18} alkyl dimethyl amine oxides and C_8-C_{12} alkoxy ethyl dihydroxy ethyl amine oxides.

When included therein, the laundry detergent compositions of the present invention typically comprise from 0.2% to about 15%, preferably from about 1% to about 10% by weight of such semi-polar nonionic surfactants.

Builder system

The compositions according to the present invention may further comprise a builder system. Any conventional builder system is suitable for use herein including aluminosilicate materials, silicates, polycarboxylates and fatty acids, materials such as ethylenediamine tetraacetate, metal ion sequestrants such as aminopolyphosphonates, particularly ethylenediamine tetramethylene phosphonic acid and diethylene triamine pentamethylenephosphonic acid. Though less preferred for obvious environmental reasons, phosphate builders can also be used herein.

Suitable builders can be an inorganic ion exchange material, commonly an inorganic hydrated aluminosilicate material, more particularly a hydrated synthetic zeolite such as hydrated zeolite A, X, B, HS or MAP.

Another suitable inorganic builder material is layered silicate, e.g. SKS-6 (Hoechst). SKS-6 is a crystalline layered silicate consisting of sodium silicate ($Na_2Si_2O_5$).

Suitable polycarboxylates containing one carboxy group include lactic acid, glycolic acid and ether derivatives thereof as disclosed in Belgian Patent Nos. 831,368, 821,369 and 821,370. Polycarboxylates containing two carboxy groups include the water-soluble salts of succinic acid, malonic acid, (ethylenedioxy) diacetic acid, maleic acid, diglycollic acid, tartaric acid, tartronic acid and fumaric acid, as well as the ether carboxylates described in German Offenlenschrift 2,446,686, and 2,446,487, US 3,935,257 and the sulfinyl carboxylates described in Belgian Patent No. 840,623. Polycarboxylates containing three carboxy groups include, in particular, water-soluble citrates, aconitrates and citraconates as well as succinate derivatives such as the carboxymethyloxysuccinates described in British Patent No. 1,379,241, lactoxysuccinates described in Netherlands Application 7205873, and the oxypolycarboxylate materials such as 2-oxa-1,1,3-propane tricarboxylates described in British Patent No. 1,387,447.

Polycarboxylates containing four carboxy groups include oxydisuccinates disclosed in British Patent No. 1,261,829, 1,1,2,2,-ethane tetracarboxylates, 1,1,3,3-propane tetracarboxylates containing sulfo substituents include the sulfosuccinate derivatives disclosed in British Patent Nos. 1,398,421 and 1,398,422 and in US 3,936,448, and the sulfonated pyrolysed citrates described in British Patent No. 1,082,179, while polycarboxylates containing phosphone substituents are disclosed in British Patent No. 1,439,000.

Alicyclic and heterocyclic polycarboxylates include cyclopentane-cis,cis-cis-tetracarboxylates, cyclopentadienide pentacarboxylates, 2,3,4,5-tetrahydro-furan - cis, cis, cis-tetracarboxylates, 2,5-tetrahydro-furan-cis, discarboxylates, 2,2,5,5,-tetrahydrofuran - tetracarboxylates, 1,2,3,4,5,6-

hexane - hexacarboxylates and carboxymethyl derivatives of polyhydric alcohols such as sorbitol, mannitol and xylitol. Aromatic polycarboxylates include mellitic acid, pyromellitic acid and the phthalic acid derivatives disclosed in British Patent No. 1,425,343.

Of the above, the preferred polycarboxylates are hydroxy-carboxylates containing up to three carboxy groups per molecule, more particularly citrates.

Preferred builder systems for use in the present compositions include a mixture of a water-insoluble aluminosilicate builder such as zeolite A or of a layered silicate (SKS-6), and a water-soluble carboxylate chelating agent such as citric acid.

A suitable chelant for inclusion in the detergent compositions in accordance with the invention is ethylenediamine-N,N'-disuccinic acid (EDDS) or the alkali metal, alkaline earth metal, ammonium, or substituted ammonium salts thereof, or mixtures thereof. Preferred EDDS compounds are the free acid form and the sodium or magnesium salt thereof. Examples of such preferred sodium salts of EDDS include Na_2EDDS and Na_4EDDS. Examples of such preferred magnesium salts of EDDS include MgEDDS and Mg_2EDDS. The magnesium salts are the most preferred for inclusion in compositions in accordance with the invention.

Preferred builder systems include a mixture of a water-insoluble aluminosilicate builder such as zeolite A, and a water soluble carboxylate chelating agent such as citric acid.

Other builder materials that can form part of the builder system for use in granular compositions include inorganic

materials such as alkali metal carbonates, bicarbonates, silicates, and organic materials such as the organic phosphonates, amino polyalkylene phosphonates and amino polycarboxylates.

Other suitable water-soluble organic salts are the homo- or co-polymeric acids or their salts, in which the polycarboxylic acid comprises at least two carboxyl radicals separated form each other by not more than two carbon atoms.

Polymers of this type are disclosed in GB-A-1,596,756. Examples of such salts are polyacrylates of MW 2000-5000 and their copolymers with maleic anhydride, such copolymers having a molecular weight of from 20,000 to 70,000, especially about 40,000.

Detergency builder salts are normally included in amounts of from 5% to 80% by weight of the composition. Preferred levels of builder for liquid detergents are from 5% to 30%.

Enzymes

In addition to the enzyme hybrid(s) in question, detergent compositions of the invention may comprise other enzymes which provide cleaning performance and/or fabric care benefits. Such enzymes include proteases, lipases, cutinases, amylases, cellulases, peroxidases and oxidases (e.g. laccases).

Proteases: Any protease suitable for use in alkaline solutions may, for example, be used. Suitable proteases include those of animal, vegetable or microbial origin. Microbial origin is preferred. Chemically or genetically modified mutants are included. The protease may be a serine protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins,

especially those derived from Bacillus, e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the Fusarium protease described in WO 89/06270.

Preferred commercially available protease enzymes include those sold under the trade names Alcalase, Savinase, Primase, Durazym, and Esperase by Novo Nordisk A/S (Denmark), those sold under the tradename Maxatase, Maxacal, Maxapem, Properase, Purafect and Purafect OXP by Genencor International, and those sold under the tradename Opticlean and Optimase by Solvay Enzymes. Protease enzymes may be incorporated into the compositions in accordance with the invention at a level of from 0.00001% to 2% of enzyme protein by weight of the composition, suitably at a level of from 0.0001% to 1% of enzyme protein by weight of the composition, such as at a level of from 0.001% to 0.5% of enzyme protein by weight of the composition, appropriately at a level of from 0.01% to 0.2% of enzyme protein by weight of the composition.

Lipases: Any lipase suitable for use in alkaline solutions may, for example, be used. Suitable lipases include those of bacterial or fungal origin. Chemically or genetically modified mutants are included.

Examples of useful lipases include a Humicola lanuginosa lipase, e.g., as described in EP 258 068 and EP 305 216, a Rhizomucor miehei lipase, e.g., as described in EP 238 023, a Candida lipase, such as a C. antarctica lipase, e.g., the C. antarctica lipase A or B described in EP 214 761, a Pseudomonas lipase such as a P. alcaligenes and P. pseudoalcaligenes lipase, e.g. as described in EP 218 272, a P. cepacia lipase, e.g., as described in EP 331 376, a P.

stutzeri lipase, e.g., as disclosed in GB 1,372,034, a P. fluorescens lipase, a Bacillus lipase, e.g., a B. subtilis lipase (Dartois et al., (1993), Biochemica et Biophysica acta 1131, 253-260), a B. stearothermophilus lipase (JP 64/744992) and a B. pumilus lipase (WO 91/16422).

Furthermore, a number of cloned lipases may be useful, including the Penicillium camembertii lipase described by Yamaguchi et al., (1991), Gene 103, 61-67), the Geotricum candidum lipase (Schimada, Y. et al., (1989), J. Biochem., 106, 383-388), and various Rhizopus lipases such as a R. delemar lipase (Hass, M.J et al., (1991), Gene 109, 117-113), a R. niveus lipase (Kugimiya et al., (1992), Biosci. Biotech. Biochem. 56, 716-719) and a R. oryzae lipase.

Other types of lipolytic enzymes such as cutinases may also be useful, e.g., a cutinase derived from Pseudomonas mendocina as described in WO 88/09367, or a cutinase derived from Fusarium solani pisi (e.g. described in WO 90/09446).

Especially suitable lipases are lipases such as M1 Lipase™, Luma fast™ and Lipomax™ (Genencor), Lipolase™ and Lipolase Ultra™ (Novo Nordisk A/S), and Lipase P "Amano" (Amano Pharmaceutical Co. Ltd.).

The lipases are normally incorporated in the detergent composition at a level of from 0.00001% to 2% of enzyme protein by weight of the composition, such as at a level of from 0.0001% to 1% of enzyme protein by weight of the composition, e.g. at a level of from 0.001% to 0.5% of enzyme protein by weight of the composition, appropriately at a level of from 0.01% to 0.2% of enzyme protein by weight of the composition.

Amylases: Any amylase (e.g. α- and/or β-) suitable for use in

alkaline solutions may, for example, be used. Suitable amylases include those of bacterial or fungal origin. Chemically or genetically modified mutants are included. Amylases include, for example, α-amylases obtained from a special strain of B. licheniformis, described in more detail in GB 1,296,839. Commercially available amylases are Duramyl™, Termamyl™, Fungamyl™ and BAN™ (available from Novo Nordisk A/S) and Rapidase™ and Maxamyl P™ (available from Genencor).

The amylases are normally incorporated in the detergent composition at a level of from 0.00001% to 2% of enzyme protein by weight of the composition, such as at a level of from 0.0001% to 1% of enzyme protein by weight of the composition, e.g. at a level of from 0.001% to 0.5% of enzyme protein by weight of the composition, appopriately at a level of from 0.01% to 0.2% of enzyme protein by weight of the composition.

Cellulases: Any cellulase suitable for use in alkaline solutions may, for example, be used. Suitable cellulases include those of bacterial or fungal origin. Chemically or genetically modified mutants are included. Suitable cellulases are disclosed in US 4,435,307, which discloses fungal cellulases produced from Humicola insolens. Especially suitable cellulases are the cellulases having colour care benefits. Examples of such cellulases are cellulases described in European patent application No. 0 495 257.

Commercially available cellulases include Celluzyme™ produced by a strain of Humicola insolens, (Novo Nordisk A/S), and KAC-500(B)™ (Kao Corporation).

Cellulases are normally incorporated in the detergent

composition at a level of from 0.00001% to 2% of enzyme protein by weight of the composition, such as at a level of from 0.0001% to 1% of enzyme protein by weight of the composition, e.g. at a level of from 0.001% to 0.5% of enzyme protein by weight of the composition, appropriately at a level of from 0.01% to 0.2% of enzyme protein by weight of the composition.

Peroxidases/oxidases: Peroxidase enzymes are normally used in combination with hydrogen peroxide or a source thereof (e.g. a percarbonate, perborate or persulfate). Oxidase enzymes are used in combination with oxygen. Both types of enzymes are used for "solution bleaching", i.e. to prevent transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, preferably together with an enhancing agent as described in e.g. WO 94/12621 and WO 95/01426. Suitable peroxidases/oxidases include those of plant, bacterial or fungal origin. Chemically or genetically modified mutants are included.

Peroxidase and/or oxidase enzymes are normally incorporated in the detergent composition at a level of from 0.00001% to 2% of enzyme protein by weight of the composition, such as at a level of from 0.0001% to 1% of enzyme protein by weight of the composition, e.g. at a level of from 0.001% to 0.5% of enzyme protein by weight of the composition, appropriately at a level of from 0.01% to 0.2% of enzyme protein by weight of the composition.

Mixtures of the above-mentioned enzymes may also be included in detergent compositions of the invention, e.g. a mixture of a protease, an amylase, a lipase and/or a cellulase.

The enzyme hybrid, or any other enzyme incorporated in the detergent composition, is normally incorporated in the

detergent composition at a level from 0.00001% to 2% of enzyme protein by weight of the composition, preferably at a level from 0.0001% to 1% of enzyme protein by weight of the composition, such as at a level of from 0.001% to 0.5% of enzyme protein by weight of the composition, e.g. at a level of from 0.01% to 0.2% of enzyme protein by weight of the composition.

Bleaching agents: Additional optional detergent ingredients that can be included in the detergent compositions of the present invention include bleaching agents such as PB1, PB4 and percarbonate with a particle size of 400-800 microns. These bleaching agent components can include one or more oxygen bleaching agents and, depending upon the bleaching agent chosen, one or more bleach activators. When present oxygen bleaching compounds will typically be present at levels of from about 1% to about 25%. In general, bleaching compounds are optional added components in non-liquid formulations, e.g. granular detergents.

A bleaching agent component for use herein can be any of the bleaching agents useful for detergent compositions including oxygen bleaches, as well as others known in the art.

A bleaching agent suitable for the present invention can be an activated or non-activated bleaching agent.

One category of oxygen bleaching agent that can be used encompasses percarboxylic acid bleaching agents and salts thereof. Suitable examples of this class of agents include magnesium monoperoxyphthalate hexahydrate, the magnesium salt of meta-chloro perbenzoic acid, 4-nonylamino-4-oxoperoxybutyric acid and diperoxydodecanedioic acid. Such bleaching agents are disclosed in US 4,483,781, US 740,446, EP 0 133 354 and US 4,412,934. Highly preferred bleaching

agents also include 6-nonylamino-6-oxoperoxycaproic acid as described in US 4,634,551.

Another category of bleaching agents that can be used encompasses the halogen bleaching agents. Examples of hypohalite bleaching agents, for example, include trichloro isocyanuric acid and the sodium and potassium dichloroisocyanurates and N-chloro and N-bromo alkane sulphonamides. Such materials are normally added at 0.5-10% by weight of the finished product, preferably 1-5% by weight.

The hydrogen peroxide releasing agents can be used in combination with bleach activators such as tetra-acetylethylenediamine (TAED), nonanoyloxybenzenesulfonate (NOBS, described in US 4,412,934), 3,5-trimethyl-hexsanoloxybenzenesulfonate (ISONOBS, described in EP 120 591) or pentaacetylglucose (PAG), which are perhydrolyzed to form a peracid as the active bleaching species, leading to improved bleaching effect. In addition, very suitable are the bleach activators C8(6-octanamido-caproyl) oxybenzene-sulfonate, C9(6-nonanamido caproyl) oxybenzenesulfonate and C10 (6-decanamido caproyl) oxybenzenesulfonate or mixtures thereof. Also suitable activators are acylated citrate esters such as disclosed in European Patent Application No. 91870207.7.

Useful bleaching agents, including peroxyacids and bleaching systems comprising bleach activators and peroxygen bleaching compounds for use in cleaning compositions according to the invention are described in application USSN 08/136,626.

The hydrogen peroxide may also be present by adding an enzymatic system (i.e. an enzyme and a substrate therefore) which is capable of generation of hydrogen peroxide at the beginning or during the washing and/or rinsing process. Such

enzymatic systems are disclosed in European Patent Application EP 0 537 381.

Bleaching agents other than oxygen bleaching agents are also known in the art and can be utilized herein. One type of non-oxygen bleaching agent of particular interest includes photoactivated bleaching agents such as the sulfonated zinc and/or aluminium phthalocyanines. These materials can be deposited upon the substrate during the washing process. Upon irradiation with light, in the presence of oxygen, such as by hanging clothes out to dry in the daylight, the sulfonated zinc phthalocyanine is activated and, consequently, the substrate is bleached. Preferred zinc phthalocyanine and a photoactivated bleaching process are described in US 4,033,718. Typically, detergent composition will contain about 0.025% to about 1.25%, by weight, of sulfonated zinc phthalocyanine.

Bleaching agents may also comprise a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

Suds suppressors: Another optional ingredient is a suds suppressor, exemplified by silicones, and silica-silicone mixtures. Silicones can generally be represented by alkylated polysiloxane materials, while silica is normally used in finely divided forms exemplified by silica aerogels and xerogels and hydrophobic silicas of various types. Theses materials can be incorporated as particulates, in which the suds suppressor is advantageously releasably incorporated in a water-soluble or water-dispersible, substantially non surface-active detergent impermeable carrier. Alternatively the suds suppressor can be dissolved or dispersed in a liquid carrier and applied by spraying on to one or more of the

other components.

A preferred silicone suds controlling agent is disclosed in US 3,933,672. Other particularly useful suds suppressors are the self-emulsifying silicone suds suppressors, described in German Patent Application DTOS 2,646,126. An example of such a compound is DC-544, commercially available form Dow Corning, which is a siloxane-glycol copolymer. Especially preferred suds controlling agent are the suds suppressor system comprising a mixture of silicone oils and 2-alkyl-alkanols. Suitable 2-alkyl-alkanols are 2-butyl-octanol which are commercially available under the trade name Isofol 12 R.

Such suds suppressor system are described in European Patent Application EP 0 593 841.

Especially preferred silicone suds controlling agents are described in European Patent Application No. 92201649.8. Said compositions can comprise a silicone/ silica mixture in combination with fumed nonporous silica such as Aerosil^{R}.

The suds suppressors described above are normally employed at levels of from 0.001% to 2% by weight of the composition, preferably from 0.01% to 1% by weight.

Other components: Other components used in detergent compositions may be employed, such as soil-suspending agents, soil-releasing agents, optical brighteners, abrasives, bactericides, tarnish inhibitors, coloring agents, and/or encapsulated or nonencapsulated perfumes.

Especially suitable encapsulating materials are water soluble capsules which consist of a matrix of polysaccharide and polyhydroxy compounds such as described in GB 1,464,616.

Other suitable water soluble encapsulating materials comprise dextrins derived from ungelatinized starch acid esters of substituted dicarboxylic acids such as described in US 3,455,838. These acid-ester dextrins are, preferably, prepared from such starches as waxy maize, waxy sorghum, sago, tapioca and potato. Suitable examples of said encapsulation materials include N-Lok manufactured by National Starch. The N-Lok encapsulating material consists of a modified maize starch and glucose. The starch is modified by adding monofunctional substituted groups such as octenyl succinic acid anhydride.

Antiredeposition and soil suspension agents suitable herein include cellulose derivatives such as methylcellulose, carboxymethylcellulose and hydroxyethylcellulose, and homo- or co-polymeric polycarboxylic acids or their salts. Polymers of this type include the polyacrylates and maleic anhydride-acrylic acid copolymers previously mentioned as builders, as well as copolymers of maleic anhydride with ethylene, methylvinyl ether or methacrylic acid, the maleic anhydride constituting at least 20 mole percent of the copolymer. These materials are normally used at levels of from 0.5% to 10% by weight, more preferably form 0.75% to 8%, most preferably from 1% to 6% by weight of the composition.

Preferred optical brighteners are anionic in character, examples of which are disodium 4,4'-bis-(2-diethanolamino-4-anilino -s- triazin-6-ylamino)stilbene-2:2' disulphonate, disodium 4, - 4'-bis-(2-morpholino-4-anilino-s-triazin-6-ylamino-stilbene-2:2' - disulphonate, disodium 4,4' - bis-(2,4-dianilino-s-triazin-6-ylamino)stilbene-2:2' - disulphonate, monosodium 4',4'' - bis-(2,4-dianilino-s-triazin-6 ylamino)stilbene-2-sulphonate, disodium 4,4' -bis-(2-anilino-4-(N-methyl-N-2-hydroxyethylamino)-s-triazin-6-ylamino)stilbene-2,2' - disulphonate, di-sodium 4,4' -bis-(4-

phenyl-2,1,3-triazol-2-yl)-stilbene-2,2' disulphonate, di-sodium 4,4'bis(2-anilino-4-(1-methyl-2-hydroxyethylamino)-s-triazin-6-ylami-no)stilbene-2,2'disulphonate, sodium 2(stilbyl-4''-(naphtho-1',2':4,5)-1,2,3, - triazole-2''-sulphonate and 4,4'-bis(2-sulphostyryl)biphenyl.

Other useful polymeric materials are the polyethylene glycols, particularly those of molecular weight 1000-10000, more particularly 2000 to 8000 and most preferably about 4000. These are used at levels of from 0.20% to 5% more preferably from 0.25% to 2.5% by weight. These polymers and the previously mentioned homo- or co-polymeric polycarboxylate salts are valuable for improving whiteness maintenance, fabric ash deposition, and cleaning performance on clay, proteinaceous and oxidizable soils in the presence of transition metal impurities.

Soil release agents useful in compositions of the present invention are conventionally copolymers or terpolymers of terephthalic acid with ethylene glycol and/or propylene glycol units in various arrangements. Examples of such polymers are disclosed in US 4,116,885 and 4,711,730 and EP 0 272 033. A particular preferred polymer in accordance with EP 0 272 033 has the formula:

$$(CH_3(PEG)_{43})_{0.75}(POH)_{0.25}[T\text{-}PO)_{2.8}(T\text{-}PEG)_{0.4}]T(POH)_{0.25}((PEG)_{43}CH_3)_{0.75}$$

where PEG is $-(OC_2H_4)O-$, PO is (OC_3H_6O) and T is $(pOOC_6H_4CO)$.

Also very useful are modified polyesters as random copolymers of dimethyl terephthalate, dimethyl sulfoisophthalate, ethylene glycol and 1,2-propanediol, the end groups consisting primarily of sulphobenzoate and secondarily of mono esters of ethylene glycol and/or 1,2-propanediol. The target is to obtain a polymer capped at both end by

sulphobenzoate groups, "primarily", in the present context most of said copolymers herein will be endcapped by sulphobenzoate groups. However, some copolymers will be less than fully capped, and therefore their end groups may consist of monoester of ethylene glycol and/or 1,2-propanediol, thereof consist "secondarily" of such species.

The selected polyesters herein contain about 46% by weight of dimethyl terephthalic acid, about 16% by weight of 1,2-propanediol, about 10% by weight ethylene glycol, about 13% by weight of dimethyl sulfobenzoic acid and about 15% by weight of sulfoisophthalic acid, and have a molecular weight of about 3.000. The polyesters and their method of preparation are described in detail in EP 311 342.

Softening agents: Fabric softening agents can also be incorporated into laundry detergent compositions in accordance with the present invention. These agents may be inorganic or organic in type. Inorganic softening agents are exemplified by the smectite clays disclosed in GB-A-1 400898 and in US 5,019,292. Organic fabric softening agents include the water insoluble tertiary amines as disclosed in GB-A1 514 276 and EP 0 011 340 and their combination with mono C_{12}-C_{14} quaternary ammonium salts are disclosed in EP-B-0 026 528 and di-long-chain amides as disclosed in EP 0 242 919. Other useful organic ingredients of fabric softening systems include high molecular weight polyethylene oxide materials as disclosed in EP 0 299 575 and 0 313 146.

Levels of smectite clay are normally in the range from 5% to 15%, more preferably from 8% to 12% by weight, with the material being added as a dry mixed component to the remainder of the formulation. Organic fabric softening agents such as the water-insoluble tertiary amines or dilong chain amide materials are incorporated at levels of from 0.5% to 5%

by weight, normally from 1% to 3% by weight whilst the high molecular weight polyethylene oxide materials and the water soluble cationic materials are added at levels of from 0.1% to 2%, normally from 0.15% to 1.5% by weight. These materials are normally added to the spray dried portion of the composition, although in some instances it may be more convenient to add them as a dry mixed particulate, or spray them as molten liquid on to other solid components of the composition.

Polymeric dye-transfer inhibiting agents: The detergent compositions according to the present invention may also comprise from 0.001% to 10%, preferably from 0.01% to 2%, more preferably form 0.05% to 1% by weight of polymeric dye-transfer inhibiting agents. Said polymeric dye-transfer inhibiting agents are normally incorporated into detergent compositions in order to inhibit the transfer of dyes from colored fabrics onto fabrics washed therewith. These polymers have the ability of complexing or adsorbing the fugitive dyes washed out of dyed fabrics before the dyes have the opportunity to become attached to other articles in the wash.

Especially suitable polymeric dye-transfer inhibiting agents are polyamine N-oxide polymers, copolymers of N-vinyl-pyrrolidone and N-vinylimidazole, polyvinylpyrrolidone polymers, polyvinyloxazolidones and polyvinylimidazoles or mixtures thereof.

Addition of such polymers also enhances the performance of the enzymes according the invention.

The detergent composition according to the invention can be in the form of a liquid, paste, gel, bar or granulate (i.e. in granular form).

Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molecular weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in GB 1483591.

Granular compositions according to the present invention can also be in "compact form", i.e. they may have a relatively higher density than conventional granular detergents, i.e. form 550 to 950 g/l; in such case, the granular detergent compositions according to the present invention will contain a lower amount of "Inorganic filler salt", compared to conventional granular detergents; typical filler salts are alkaline earth metal salts of sulphates and chlorides, typically sodium sulphate; "Compact" detergent typically comprise not more than 10% filler salt. The liquid compositions according to the present invention can also be in "concentrated form", in such case, the liquid detergent compositions according to the present invention will contain a lower amount of water, compared to conventional liquid detergents. Typically, the water content of the concentrated liquid detergent is less than 30%, more preferably less than 20%, most preferably less than 10% by weight of the detergent compositions.

The compositions of the invention may, for example, be formulated as hand and machine laundry detergent compositions

including laundry additive compositions and compositions suitable for use in the pretreatment of stained fabrics.

The following examples are intended to exemplify compositions within the scope of the present invention, but are not intended to limit or otherwise define the scope of the invention. In the detergent compositions, the abbreviated component identifications have the following meanings:

LAS: Sodium linear C_{12} alkyl benzene sulphonate

TAS: Sodium tallow alkyl sulphate

XYAS: Sodium C_{1X} - C_{1Y} alkyl sulfate

SS: Secondary soap surfactant of formula 2-butyl octanoic acid

25EY: A C_{12} - C_{15} predominantly linear primary alcohol condensed with an average of Y moles of ethylene oxide

45EY: A C_{14} - C_{15} predominantly linear primary alcohol condensed with an average of Y moles of ethylene oxide

XYEZS: C_{1X} - C_{1Y} sodium alkyl sulfate condensed with an average of Z moles of ethylene oxide per mole

Nonionic: C_{13} - C_{15} mixed ethoxylated/propoxylated fatty alcohol with an average degree of ethoxylation of 3.8 and an average degree of propoxylation of 4.5 sold under the tradename Plurafax LF404 by BASF Gmbh

CFAA: C_{12} - C_{14} alkyl N-methyl glucamide

TFAA: C_{16} - C_{18} alkyl N-methyl glucamide

Silicate: Amorphous Sodium Silicate (SiO_2:Na_2O ratio = 2.0)

NaSKS-6: Crystalline layered silicate of formula δ-$Na_2Si_2O_5$

Carbonate: Anhydrous sodium carbonate

Phosphate: Sodium tripolyphosphate

MA/AA: Copolymer of 1:4 maleic/acrylic acid, average molecular weight about 80,000

Polyacrylate: Polyacrylate homopolymer with an average molecular weight of 8,000 sold under the tradename PA30 by BASF Gmbh

Zeolite A: Hydrated Sodium Aluminosilicate of formula $Na_{12}(AlO_2SiO_2)_{12}.27H_2O$ having a primary particle size in the range from 1 to 10 micrometers

Citrate: Tri-sodium citrate dihydrate

Citric: Citric Acid

Perborate: Anhydrous sodium perborate monohydrate bleach, empirical formula $NaBO_2.H_2O_2$

PB4: Anhydrous sodium perborate tetrahydrate

Percarbonate: Anhydrous sodium percarbonate bleach of empirical formula $2Na_2CO_3.3H_2O_2$

TAED: Tetraacetyl ethylene diamine

CMC: Sodium carboxymethyl cellulose

DETPMP: Diethylene triamine penta (methylene phosphonic acid), marketed by Monsanto under the Tradename Dequest 2060

PVP: Polyvinylpyrrolidone polymer

EDDS: Ethylenediamine-N, N'-disuccinic acid, [S,S] isomer in the form of the sodium salt

Suds 25% paraffin wax Mpt 50°C, 17% hydrophobic silica, 58%

Suppressor: paraffin oil

Granular Suds 12% Silicone/silica, 18% stearyl alcohol, 70%
suppressor: starch in granular form

Sulphate: Anhydrous sodium sulphate

HMWPEO: High molecular weight polyethylene oxide

TAE 25: Tallow alcohol ethoxylate (25)

In the following compositions, "Enzyme" refers to enzyme hybrid(s) and any added enzyme(s):

Detergent Example I

A granular fabric cleaning composition in accordance with the invention may be prepared as follows:

Sodium linear C_{12} alkyl benzene sulfonate	6.5
Sodium sulfate	15.0
Zeolite A	26.0
Sodium nitrilotriacetate	5.0
Enzyme	0.1
PVP	0.5
TAED	3.0
Boric acid	4.0
Perborate	18.0
Phenol sulphonate	0.1
Minors	Up to 100

Detergent Example II

A compact granular fabric cleaning composition (density 800 g/l) in accord with the invention may be prepared as follows:

45AS	8.0
25E3S	2.0
25E5	3.0
25E3	3.0
TFAA	2.5
Zeolite A	17.0
NaSKS-6	12.0
Citric acid	3.0
Carbonate	7.0
MA/AA	5.0
CMC	0.4
Enzyme	0.1

TAED	6.0
Percarbonate	22.0
EDDS	0.3
Granular suds suppressor	3.5
water/minors	Up to 100%

Detergent Example III

Granular fabric cleaning compositions in accordance with the invention which are useful in the laundering of coloured fabrics may be prepared as follows:

LAS	10.7	-
TAS	2.4	-
TFAA	-	4.0
45AS	3.1	10.0
45E7	4.0	-
25E3S	-	3.0
68E11	1.8	-
25E5	-	8.0
Citrate	15.0	7.0
Carbonate	-	10
Citric acid	2.5	3.0
Zeolite A	32.1	25.0
Na-SKS-6	-	9.0
MA/AA	5.0	5.0
DETPMP	0.2	0.8
Enzyme	0.10	0.05
Silicate	2.5	-
Sulphate	5.2	3.0
PVP	0.5	-
Poly (4-vinylpyridine)-N-Oxide/copolymer of vinyl-imidazole and vinyl-pyrrolidone	-	0.2
Perborate	1.0	-
Phenol sulfonate	0.2	-

Water/Minors	Up to 100%	

Detergent Example IV

Granular fabric cleaning compositions in accordance with the invention which provide "Softening through the wash" capability may be prepared as follows:

45AS	-	10.0
LAS	7.6	-
68AS	1.3	-
45E7	4.0	-
25E3	-	5.0
Coco-alkyl-dimethyl hydroxy-ethyl ammonium chloride	1.4	1.0
Citrate	5.0	3.0
Na-SKS-6	-	11.0
Zeolite A	15.0	15.0
MA/AA	4.0	4.0
DETPMP	0.4	0.4
Perborate	15.0	-
Percarbonate	-	15.0
TAED	5.0	5.0
Smectite clay	10.0	10.0
HMWPEO	-	0.1
Enzyme	0.10	0.05
Silicate	3.0	5.0
Carbonate	10.0	10.0
Granular suds suppressor	1.0	4.0
CMC	0.2	0.1
Water/Minors	Up to 100%	

Detergent Example V

Heavy duty liquid fabric cleaning compositions in accordance with the invention may be prepared as follows:

	I	II
LAS acid form	-	25.0
Citric acid	5.0	2.0
25AS acid form	8.0	-
25AE2S acid form	3.0	-
25AE7	8.0	-
CFAA	5	-
DETPMP	1.0	1.0
Fatty acid	8	-
Oleic acid	-	1.0
Ethanol	4.0	6.0
Propanediol	2.0	6.0
Enzyme	0.10	0.05
Coco-alkyl dimethyl hydroxy ethyl ammonium chloride	-	3.0
Smectite clay	-	5.0
PVP	2.0	-
Water / Minors	Up to 100%	

The enzyme hybrid may be incorporated in concentrations conventionally employed in detergents. It is at present contemplated that, in the detergent composition of the invention, the enzyme hybrid may suitably be added in an amount corresponding to 0.00001-1 mg (calculated as pure enzymatic protein) of enzyme hybrid per liter of wash liquor.

Reaction time

The reaction time for removing or bleaching the soiling or stain(s) from fabric may vary; the fabric may be soaked for one or two days, or the washing may be performed within a shorter period, typically machine-washed for a period of 1 to 90 minutes, preferably for a period of 1 to 30 minutes.

A further aspect of the invention relates to a DNA construct disclosed herein which encodes, or which comprises a sequence which encodes, an enzyme hybrid as disclosed in the present specification.

A still further aspect of the invention relates to a polypeptide (fusion protein or enzyme hybrid) which is encoded by such a DNA construct or sequence, and/or which is disclosed in the present specification. Thus, the invention encompasses an enzyme hybrid encoded by a hybrid-encoding DNA sequence comprised within the DNA sequences of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18 or SEQ ID No. 19, or an enzyme hybrid having an amino acid sequence comprised within the amino acid sequences of SEQ ID No. 2, SEQ ID No. 4, SEQ ID No. 6 or SEQ ID No. 8.

The invention is further illustrated in the following example, which are not intended to be in any way limiting to the scope of the invention as claimed.

MATERIALS AND METHODS

Strains:

Bacillus agaradherens NCIMB No. 40482: comprises the endoglucanase enzyme encoding DNA sequence of Example 2, below.

Escherichia coli SJ2 [Diderichsen et al., J. Bacteriol. 172 (1990), pp. 4315-4321].

Electrocompetent cells prepared and transformed using a Bio-Rad GenePulser™ as recommended by the manufacturer.

Bacillus subtilis PL2306: this strain is the *B.subtilis* DN1885 with disrupted apr and npr genes [Diderichsen et al., J. Bacteriol. 172 (1990), pp. 4315-4321] disrupted in the transcriptional unit of the known *Bacillus subtilis* cellulase gene, resulting in cellulase-negative cells. The disruption was performed essentially as described in Sonenshein et al. (Eds.), *Bacillus subtilis* and other Gram-Positive Bacteria, American Society for Microbiology (1993), p.618.

Plasmids:

pDN1528 [Jørgensen et al., J. Bacteriol. 173 (1991), p.559-567].

pBluescriptKSII- (Stratagene, USA).

pDN1981 [Jørgensen et al., Gene 96 (1990), p37-41].

Solutions/Media

TY and LB agar [as described in Ausubel et al. (Eds.), Current Protocols in Molecular Biology, John Wiley and Sons (1995)].

SB: 32 g Tryptone, 20 g yeast extract, 5 g sodium chloride and 5 ml 1 N sodium hydroxide are mixed in sterile water to a final volume of 1 litre. The solution is sterilised by autoclaving for 20 minutes at 121°C.

10% Avicel™: 100 g of Avicel™ (FLUKA, Switzerland) is mixed with sterile water to a final volume of 1 litre, and the resulting 10% Avicel™ is sterilised by autoclaving for 20

minutes at 121°C.

Buffer: 0.05 M potassium phosphate, pH 7.5.

General molecular biology methods

DNA manipulations and transformations were performed using standard methods of molecular biology [Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor lab., Cold Spring Harbor, NY (1989); Ausubel et al. (Eds.), Current Protocols in Molecular Biology, John Wiley and Sons (1995); C.R. Harwood and S.M. Cutting (Eds.) Molecular Biological Methods for Bacillus, John Wiley and Sons (1990)].

Enzymes for DNA manipulations were used according to the specifications of the suppliers.

EXAMPLE 1

Subcloning of a partial Termamyl sequence.

The alfa-amylase gene encoded on pDN1528 was PCR amplified for introduction of a BamHI site in the 3'-end of the coding region. The PCR and the cloning were carried out as follows:

Approximately 10-20 ng of plasmid pDN1528 was PCR amplified in HiFidelity™ PCR buffer (Boehringer Mannheim, Germany) supplemented with 200 µM of each dNTP, 2.6 units of HiFidelity™ Expand enzyme mix, and 300 pmol of each primer:

#5289
5'-GCT TTA CGC CCG ATT GCT GAC GCT G -3'

#26748
5'-GCG ATG AGA CGC GCG GCC GCC TAT CTT TGA ACA TAA ATT GAA

ACG GAT CCG -3'

(BamHI restriction site underlined].

The PCR reactions were performed using a DNA thermal cycler (Landgraf, Germany). One incubation at 94°C for 2 min, 60°C for 30 sec and 72°C for 45 sec was followed by ten cycles of PCR performed using a cycle profile of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 45sec and twenty cycles of denaturation at 94°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec (at this elongation step, 20 sec are added every cycle). 10 µl aliquots of amplification product were analyzed by electrophoresis in 1.0 % agarose gels (NuSieve™, FMC) with ReadyLoad™ 100bp DNA ladder (GibcoBRL, Denmark) as a size marker.

40 µl aliquots of PCR product generated as described above were purified using QIAquick™ PCR purification kit (Qiagen, USA) according to the manufacturer's instructions. The purified DNA was eluted in 50 µl of 10mM Tris-HCl, pH 8.5. 25 µl of the purified PCR fragment was digested with BamHI and PstI, subjected to electrophoresis in 1.0% low gelling temperature agarose (SeaPlaque™ GTG, FMC) gels, and the relevant fragment was excised from the gel and purified using QIAquick™ Gel extraction Kit (Qiagen, USA) according to the manufacturer's instructions. The isolated DNA fragment was then ligated to BamHI-PstI digested pBluescriptII KS-, and the ligation mixture was used to transform *E. coli* SJ2.

Cells were plated on LB agar plates containing Ampicillin (200 µg/ml) and supplemented with X-gal (5-bromo-4-chloro-3-

indolyl-α-D-galactopyranoside, 50 µg/ml), and incubated at 37°C overnight. The next day, white colonies were restreaked onto fresh LB-Ampicillin agar plates and incubated at 37°C overnight. The following day, single colonies were transferred to liquid LB medium containing Ampicillin (200 µg/ml) and incubated overnight at 37°C with shaking at 250 rpm.

Plasmids were extracted from the liquid cultures using QIAgen Plasmid Purification mini kit (Qiagen, USA) according to the manufacturer's instructions. 5 µl samples of the plasmids were digested with PstI and BamHI. The digestions were checked by gel electrophoresis on a 1.0% agarose gel (NuSieve™, FMC). One positive clone, containing the PstI-BamHI fragment containing part of the α-amylase gene, was designated pMB335. This plasmid was then used in the construction of α-amylase-CBD hybrid.

Isolation of genomic DNA

Clostridium stercorarium NCIMB 11754 was grown anaerobically at 60°C in specified media as recommended by The National Collections of Industrial and Marine Bacteria Ltd. (NCIMB), Scotland. Cells were harvested by centrifugation.

Genomic DNA was isolated as described by Pitcher et al, Lett. Appl. Microbiol. 8 (1989), pp. 151-156.

In vitro amplification of the CBD-dimer of *Clostridium stercorarium* (NCIMB 11754) XynA

Approximately 100-200 ng of genomic DNA was PCR amplified in HiFidelity™ PCR buffer (Boehringer Mannheim, Germany) supplemented with 200 µM of each dNTP, 2.6 units of HiFidelity™ Expand enzyme mix, and 300 pmol of each primer:

#27183
5'-GCT GCA GGA TCC GTT TCA ATT TAT GTT CAA AGA TCT GGC GGA CCT GGA ACG CCA AAT AAT GGA AGA GG -3'

#27182
5'-GCA CTA GCT AGA CGG CCG CTA CCA GTC AAC ATT AAC AGG ACC TGA G -3'

(BamHI and EagI restriction sites underlined).

The primers were designed to amplify the DNA encoding the cellulose-binding domain of the XynA-encoding gene of *Clostridium stercorarium* NCIMB 11754; the DNA sequence was extracted from the database GenBank under the accession number D13325.

The PCR reactions were performed using a DNA thermal cycler (Landgraf, Germany). One incubation at 94°C for 2 min, 60°C for 30 sec and 72°C for 45 sec was followed by ten cycles of PCR performed using a cycle profile of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 45 sec and twenty cycles of denaturation at 94°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec (at this elongation step, 20 sec are added every cycle). 10 µl aliquots of amplification product were analyzed by electrophoresis in 1.0 % agarose gels (NuSieve™, FMC) with ReadyLoad™ 100bp DNA ladder (GibcoBRL, Denmark) as a size marker.

Cloning by polymerase chain reaction (PCR):
Subcloning of PCR fragments.

40 µl aliquots of PCR product generated as described above

were purified using QIAquick™ PCR purification kit (Qiagen, USA) according to the manufacturer's instructions. The purified DNA was eluted in 50 µl of 10mM Tris-HCl, pH 8.5. 25 µl of the purified PCR fragment was digested with BamHI and EagI, subjected to electrophoresis in 1.0% low gelling temperature agarose (SeaPlaque™ GTG, FMC) gels, and the relevant fragment was excised from the gels and purified using QIAquick™ Gel extraction Kit (Qiagen, USA) according to the manufacturer's instructions. The isolated DNA fragment was then ligated to BamHI-NotI digested pMB335 and the ligation mixture was used to transform *E.coli* SJ2.

Identification and characterization of positive clones

Cells were plated on LB agar plates containing Ampicillin (200 µg/ml) and incubated at 37°C overnight. The next day, colonies were restreaked onto fresh LB-Ampicillin agar plates and incubated at 37°C overnight. The following day, single colonies were transferred to liquid LB medium containing Ampicillin (200 µg/ml) and incubated overnight at 37°C with shaking at 250 rpm.

Plasmids were extracted from the liquid cultures using QIAgen Plasmid Purification mini kit (Qiagen, USA) according to the manufacturer's instructions. 5 µl samples of the plasmids were digested with BamHI and NotII. The digestions were checked by gel electrophoresis on a 1.0% agarose gel (NuSieve™, FMC). The appearance of a DNA fragment of the same size as seen from the PCR amplification indicated a positive clone.

One positive clone, containing the fusion construct of the α-amylase gene and the CBD-dimer of *Clostridium stercorarium* (NCIMB 11754) XynA, was designated MBamyX.

Cloning of the fusion construct into a *Bacillus*-based expression vector

The pDN1528 vector contains the amyL gene of *B.licheniformis*; this gene is actively expressed in *B. subtilis*, resulting in the production of active α-amylase appearing in the supernatant. For expression purposes, the DNA encoding the fusion protein as constructed above was introduced to pDN1528.

This was done by digesting pMBamyX and pDN1528 with SalI-NotI, purifying the fragments and ligating the 4.7 kb pDN1528 SalI-NotI fragment with the 1.0 kb pMBamyX SalI-NotI fragment. This created an inframe fusion of the hybrid construction with the Termamyl™ (*B. licheniformis* α-amylase) gene. The DNA sequence of the fusion construction of pMB206, and the corresponding amino acid sequence, are shown in SEQ ID No. 1 and SEQ ID No. 2, respectively.

The ligation mixture was used to transform competent cells of *B. subtilis* PL2306. Cells were plated on LB agar plates containing chloramphenicol (6 µg/ml), 0.4% glucose and 10mM potassium hydrogen phosphate, and incubated at 37°C overnight. The next day, colonies were restreaked onto fresh LBPG (LB plates with 0.4% glucose and 10mM potassium phosphate, pH 10) chloramphenicol agar plates and incubated at 37°C overnight. The following day, single colonies of each clone were transferred to liquid LB medium containing chloramphenicol (6 µg/ml) and incubated overnight at 37°C with shaking at 250 rpm.

Plasmids were extracted from the liquid cultures using QIAgen Plasmid Purification mini kit (Qiagen, USA) according to the manufacturer's instructions. However, the resuspension buffer was supplemented with 1 mg/ml of chicken egg white lysozyme

(SIGMA, USA) prior to lysing the cells at 37°C for 15 minutes. 5 µl samples of the plasmids were digested with BamHI and NotI. The digestions were checked by gel electrophoresis on a 1.5% agarose gel (NuSieve™, FMC). The appearance of a DNA fragment of the same size as seen from the PCR amplification indicated a positive clone. One positive clone was designated MB-BSamyx.

Expression, secretion and functional analysis of the fusion protein

The clone MB-BSamyx (expressing Termamyl™ fused to *C.stercorarium* XynA dimer CBD) was incubated for 20 hours in SB medium at 37°C with shaking at 250 rpm. 1 ml of cell-free supernatant was mixed with 200 µl of 10% Avicel™. The mixture was incubated for 1 hour at 0°C and then centrifuged for 5 minutes at 5000 x g. The pellet was resuspended in 100 µl of SDS-PAGE buffer, and the suspension was boiled at 95°C for 5 minutes, centrifuged at 5000 x g for 5 minutes, and 25 µl was loaded onto a 4-20% Laemmli Tris-Glycine, SDS-PAGE NOVEX™ gel (Novex, USA). The samples were subjected to electrophoresis in an Xcell™ Mini-Cell (NOVEX, USA) as recommended by the manufacturer. All subsequent handling of gels, including staining (Coomassie), destaining and drying, were performed as described by the manufacturer.

The appearance of a protein band of molecular weight approx. 85 kDa indicated expression in *B.subtilis* of the Termamyl-CBD fusion amyx.

EXAMPLE 2

Identification of a novel CBD representing a new CBD family

The alkaline cellulase cloned in *Bacillus subtilis* as

described below was expressed by incubating the clone for 20 hours in SB medium at 37°C with shaking at 250 rpm. The expressed cellulase was shown to contain a CBD by its ability to specifically bind to Avicel™.

When left to incubate for a further 20 hours, the cellulase was proteolytically cleaved and two specific protein bands appeared in SDS-PAGE, one corresponding to the catalytic part of the cellulase, approximate molecular weight (MW) 35 kD, and the other corresponding to a proposed linker and CBD of approximate MW 8 kD.

The CBD was found to be the C-terminal part of the cellulase, and did not match any of the CBD families described previously [Tomme et al., Cellulose-Binding Domains: Classification and Properties, In: J.N. Saddler and M.H. Penner (Eds.), Enzymatic Degradation of Insoluble Carbohydrates, ACS Symposium Series No. 618 (1996)]. Accordingly, this CBD appears to be the first member of a new family.

Cloning of the alkaline cellulase (endoglucanase) from *Bacillus agaradherens* and expression of the alkaline cellulase in *Bacillus subtilis*

The nucleotide sequence encoding the alkaline cellulase from *Bacillus agaradherens* (deposited under accession No. NCIMB 40482) was cloned by PCR for introduction in an expression plasmid pDN1981. PCR was performed essentially as described above on 500 ng of genomic DNA, using the following two primers containing NdeI and KpnI restriction sites for introducing the endoglucanase-encoding DNA sequence to pDN1981 for expression:

#20887

5'-GTA GGC TCA GTC ATA TGT TAC ACA TTG AAA GGG GAG GAG AAT CAT GAA AAA GAT AAC TAC TAT TTT TGT CG-3'

#21318

5'-GTA CCT CGC GGG TAC CAA GCG GCC GCT TAA TTG AGT GGT TCC CAC GGA CCG-3'

After PCR cycling, the PCR fragment was purified using QIAquick™ PCR column kit (Qiagen, USA) according to the manufacturer's instructions. The purified DNA was eluted in 50 µl of 10mM Tris-HCl, pH 8.5, digested with NdeI and KpnI, purified and ligated to digested pDN1981. The ligation mixture was used to transform *B. subtilis* PL2306. Competent cells were prepared and transformed as described by Yasbin et al., J. Bacteriol. 121 (1975), pp. 296-304.

Isolation and testing of *B. subtilis* transformants

The transformed cells were plated on LB agar plates containing Kanamycin (10 mg/ml), 0.4% glucose, 10 mM potassium phosphate and 0.1% AZCL HE-cellulose (Megazyme, Australia), and incubated at 37 °C for 18 hours. Endoglucanase-positive colonies were identified as colonies surrounded by a blue halo.

Each of the positive transformants was inoculated in 10 ml TY medium containing Kanamycin (10 mg/ml). After 1 day of incubation at 37°C with shaking at 250rpm, 50 ml of supernatant was removed. The endoglucanase activity was identified by adding 50 ml of supernatant to holes punctured in the agar of LB agar plates containing 0.1% AZCL HE-cellulose.

After 16 hours incubation at 37°C, blue halos surrounding holes indicated expression of the endoglucanase in *B. subtilis*. One such clone was designated MB208. The encoding

DNA sequence and amino acid sequence of the endoglucanase are shown in SEQ ID No. 3 and SEQ ID No. 4, respectively.

The DNA sequence was determined as follows: Qiagen purified plasmid DNA was sequenced with the Taq deoxy terminal cycle sequencing kit (Perkin Elmer, USA) using the primers #21318 and #20887 (*vide supra*) and employing an Applied Biosystems 373A automated sequencer operated according to the manufacturer's instructions. Analysis of the sequence data is performed according to Devereux et al., Carcinogenesis 14 (1993), pp. 795-801.

***In vitro* amplification of the CBD of *Bacillus agaradherens* NCIMB 40482 endoglucanase**

Approximately 10-20 ng of plasmid pMB208 was PCR amplified in HiFidelity™ PCR buffer (Boehringer Mannheim, Germany) supplemented with 200 µM of each dNTP, 2.6 units of HiFidelity™ Expand enzyme mix and 300 pmol of each primer:

#27184
5'-GCT GCA GGA TCC GTT TCA ATT TAT GTT CAA AGA TCT CCT GGA GAG TAT CCA GCA TGG GAC CCA A-3'

#28495
5'-GC ACA AGC TTG CGG CCG CTA ATT GAG TGG TTC CCA CGG ACC G -3'

(BamHI and NotI restriction sites underlined).

The primers were designed to amplify the CBD-encoding DNA of the cellulase-encoding gene of *Bacillus agaradherens* NCIMB 40482.

The PCR reaction was performed using a DNA thermal cycler (Landgraf, Germany). One incubation at 94°C for 2 min, 60°C for 30 sec and 72°C for 45 sec was followed by ten cycles of PCR performed using a cycle profile of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 45 sec and twenty cycles of denaturation at 94°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec (at this elongation step, 20 sec are added every cycle). 10 µl aliquots of amplification product were analyzed by electrophoresis in 1.5 % agarose gels (NuSieve™, FMC) with ReadyLoad™ 100bp DNA ladder (GibcoBRL, Denmark) as a size marker.

Cloning by polymerase chain reaction (PCR):

Subcloning of PCR fragments

40 µl aliquots of PCR products generated as described above were purified using QIAquick™ PCR purification kit (Qiagen, USA) according to the manufacturer's instructions. The purified DNA was eluted in 50 µl of 10mM Tris-HCl, pH 8.5. 25 µl of the purified PCR fragment was digested with BamHI and NotI, subjected to electrophoresis in 1.5% low gelling temperature agarose (SeaPlaque™ GTG, FMC) gels, and the relevant fragment was excised from the gels and purified using QIAquick™ Gel extraction kit (Qiagen, USA) according to the manufacturer's instructions. The isolated DNA fragment was then ligated to BamHI-NotI digested pMB335, and the ligation mixture was used to transform *E. coli* SJ2.

Identification and characterization of positive clones

Cells were plated on LB agar plates containing Ampicillin (200 µg/ml) and incubated at 37°C overnight. The next day, colonies were restreaked onto fresh LB-Ampicillin agar plates and incubated at 37°C overnight. The following day, single

colonies were transferred to liquid LB medium containing Ampicillin (200 µg/ml) and incubated overnight at 37°C with shaking at 250 rpm.

Plasmids were extracted from the liquid cultures using QIAgen Plasmid Purification mini kit (Qiagen, USA) according to the manufacturer's instructions. 5 µl samples of the plasmids were digested with BamHI and NotI. The digestions were checked by gel electrophoresis on a 1.5% agarose gel (NuSieve™, FMC). The appearance of a DNA fragment of the same size as seen from the PCR amplification indicated a positive clone.

One positive clone, containing the fusion construct of the Termamyl™ α-amylase gene and the CBD of *Bacillus agaradherens* NCIMB 40482 alkaline cellulase Cel5A, was designated MBamyC5A.

Cloning of the fusion construct into a *Bacillus*-based expression vector

As mentioned previously, the amyL gene of *B. licheniformis* (contained in the pDN1528 vector) is actively expressed in *B. subtilis*, resulting in the production of active α-amylase appearing in the supernatant. For expression purposes, the DNA encoding the fusion protein as constructed above was introduced to pDN1528. This was done by digesting pMBamyC5A and pDN1528 with SalI-NotI, purifying the fragments and ligating the 4.7 kb pDN1528 SalI-NotI fragment with the 0.5 kb pMBamyC5A SalI-NotI fragment. This created an inframe fusion of the hybrid construction with the Termamyl™ gene. The DNA sequence of the fusion construction of pMB378, and the corresponding amino acid sequence, are shown in SEQ ID No. 5 and SEQ ID No. 6, respectively.

The ligation mixture was used to transform competent cells of *B. subtilis* PL2306. Cells were plated on LB agar plates containing chloramphenicol (6 µg/ml), 0.4% glucose and 10mM potassium hydrogen phosphate, and incubated at 37°C overnight. The next day, colonies were restreaked onto fresh LBPG chloramphenicol agar plates and incubated at 37°C overnight. The following day, single colonies of each clone were transferred to liquid LB medium containing chloramphenicol (6 µg/ml) and incubated overnight at 37°C with shaking at 250 rpm.

Plasmids were extracted from the liquid cultures using QIAgen Plasmid Purification mini kit (Qiagen, USA) according to the manufacturer's instructions. However, the resuspension buffer was supplemented with 1 mg/ml of chicken egg white lysozyme (SIGMA, USA) prior to lysing the cells at 37°C for 15 minutes. 5 µl samples of the plasmids were digested with BamHI and NotI. The digestions were checked by gel electrophoresis on a 1.5% agarose gel (NuSieve™, FMC). The appearance of a DNA fragment of the same size as seen from the PCR amplification indicated a positive clone. One positive clone was designated MB378.

Expression, secretion and functional analysis of the fusion protein

The clone MB378 (expressing Termamyl™ fused to *Bacillus agaradherens* Cel5A CBD) was incubated for 20 hours in SB medium at 37°C with shaking at 250 rpm. 1 ml of cell-free supernatant was mixed with 200 µl of 10% Avicel™. The mixture was incubated for 1 hour at 0°C and then centrifuged for 5 minutes at 5000 x g. The pellet was resuspended in 100 µl of SDS-PAGE buffer, and the suspension was boiled at 95°C

for 5 minutes, centrifuged at 5000 x g for 5 minutes, and 25 µl was loaded onto a 4-20% Laemmli Tris-Glycine, SDS-PAGE NOVEX™ gel (Novex, USA). The samples were subjected to electrophoresis in an Xcell™ Mini-Cell (NOVEX, USA) as recommended by the manufacturer. All subsequent handling of gels, including staining (Coomassie), destaining and drying, were performed as described by the manufacturer.

The appearance of a protein band of molecular weight approx. 60 kDa indicated expression in *B. subtilis* of the Termamyl™-CBD fusion encoded on the plasmid pMB378.

EXAMPLE 3

This example describes fusion of Termamyl™ and the CBD from *Cellulomonas fimi* (ATCC484) *cenA* gene using the sequence overlap extension (SOE) procedure [see, e.g., Sambrook et al., Ausubel et al., or C.R. Harwood and S.M. Cutting (*loc. cit.*)]. The final construction is as follows: Termamyl™ promoter - Termamyl™ signal peptide - cenA CBD - linker - mature Termamyl™.

Amplification of the Termamyl™ fragment for SOE

Approximately 10-20 ng of plasmid pDN1528 was PCR amplified in HiFidelity™ PCR buffer (Boehringer Mannheim, Germany) supplemented with 200 µM of each dNTP, 2.6 units of HiFidelity™ Expand enzyme mix, and 100 pmol of each primer:

#4576
5'-CTC GTC CCA ATC GGT TCC GTC -3´

#28403
5'-TGC ACT GGT ACA GTT CCT ACA ACT AGT CCT ACA CGT GCA AAT CTT AAT GGG ACG CTG -3'

The part of the primer #28403 constituting a fragment of the Termamyl™ sequence is underlined. The sequence on the 5' side of this underlined sequence is that coding for the linker region to the CBD.

The PCR reaction was performed using a DNA thermal cycler (Landgraf, Germany). One incubation at 94°C for 2 min, 55°C for 30 sec and 72°C for 45 sec was followed by twenty cycles of PCR performed using a cycle profile of denaturation at 96°C for 10 sec, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec. 10 µl aliquots of the amplification product were analyzed by electrophoresis in 1.0 % agarose gels (NuSieve™, FMC) with ReadyLoad™ 100bp DNA ladder (GibcoBRL, Denmark) as a size marker.

40 µl aliquots of the PCR product generated as described above were purified using QIAquick™ PCR purification kit (Qiagen, USA) according to the manufacturer's instructions. The purified DNA was eluted in 50 µl of 10mM Tris-HCl, pH 8.5.

Isolation of genomic DNA

Cellulomonas fimi ATCC484 was grown in TY medium at 30°C with shaking at 250 rpm for 24 hours. Cells were harvested by centrifugation.

Genomic DNA was isolated as described by Pitcher et al., Lett. Appl. Microbiol. 8 (1989), pp. 151-156.

***In vitro* amplification of the CBD of *Cellulomonas fimi* (ATCC484) *cenA* gene for SOE procedure**

Approximately 100-200 ng of genomic DNA was PCR amplified in HiFidelity™ PCR buffer (Boehringer Mannheim, Germany) supplemented with 200 µM of each dNTP, 2.6 units of HiFidelity™ Expand enzyme mix, and 100 pmol of each primer:

#8828
5'-CTG CCT CAT TCT GCA GCA GCG GCG GCA AAT CTT AAT GCT CCC GGC TGC CGC GTC GAC TAC -3'

#28404
5'-TGT AGG AAC TGT ACC AGT GCA CGT GGT GCC GTT GAG C -3'

(PstI restriction site underlined).

The primers were designed to amplify the DNA encoding the cellulose-binding domain of the CenA-encoding gene of *Cellulomonas fimi* (ATCC484). The DNA sequence was extracted from the database GenBank under the accession number M15823.

PCR cycling was performed as follows: One incubation at 94°C for 2 min, 55°C for 30 sec and 72°C for 45 sec was followed by thirty cycles of PCR performed using a cycle profile of denaturation at 96°C for 10 sec, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec. 10 µl aliquots of the amplification product were analyzed by electrophoresis in 1.0 % agarose gels (NuSieve™, FMC) with ReadyLoad™ 100bp DNA ladder (GibcoBRL, Denmark) as a size marker.

40 µl aliquots of the PCR product generated as described above were purified using QIAquick™ PCR purification kit (Qiagen, USA) according to the manufacturer's instructions.

The purified DNA was eluted in 50 µl of 10mM Tris-HCl, pH 8.5.

SOE of the CBD from *Cellulomonas fimi* (ATCC484) *cenA* gene and the Termamyl™ gene

Approximately 100-200 ng of the PCR amplified Termamyl™ fragment and the PCR amplified *cenA* CBD fragment were used in a second round of PCR. SOE of the two fragments was performed in in HiFidelity™ PCR buffer (Boehringer Mannheim, Germany) supplemented with 200 µM of each dNTP, 2.6 units of HiFidelity™ Expand enzyme mix.

A touch-down PCR cycling was performed as follows: One incubation at 96°C for 2 min, 60°C for 2 min and 72°C for 45 sec. This cycle was repeated ten times with a 1°C decrease of the annealing temperature at each cycle.

A third PCR reaction was started by adding 100 pmol of the two flanking primers #8828 and #4576 (*vide supra*) to amplify the hybrid DNA. PCR was performed by incubating the SOE reaction mixture at 96°C for 2 min, 55°C for 30 sec and 72°C for 45 sec. This was followed by twenty cycles of PCR performed using a cycle profile of denaturation at 96°C for 10 sec, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec. 10 µl aliquots of the amplification product were analyzed by electrophoresis in 1.0 % agarose gels (NuSieve™, FMC) with ReadyLoad™ 100bp DNA ladder (GibcoBRL, Denmark) as a size marker. The SOE fragment had the expected size of 879 bp.

Subcloning of the SOE fragment coding for the CBD-Termamyl

hybrid

40 µl of the SOE-PCR product generated as described above was purified using QIAquick™ PCR purification kit (Qiagen, USA) according to the manufacturer's instructions. The purified DNA was eluted in 50 µl of 10mM Tris-HCl, pH 8.5. 25 µl of the purified PCR fragment was digested with PstI and KpnI, subjected to electrophoresis in 1.0% low gelling temperature agarose (SeaPlaque™ GTG, FMC) gels, and a fragment of 837 bp was excised from the gel and purified using QIAquick™ Gel extraction Kit (Qiagen, USA) according to the manufacturer's instructions. The isolated DNA fragment was then ligated to PstI- and KpnI-digested pDN1981, and the ligation mixture was used to transform competent cells of *B. subtilis* PL2306. Cells were plated on LB agar plates containing Kanamycin (10 µg/ml), 0.4% glucose and 10mM potassium hydrogen phosphate, and incubated at 37°C overnight. The next day, colonies were restreaked onto fresh LBPG Kanamycin agar plates and incubated at 37°C overnight. The following day, single colonies of each clone were transferred to liquid LB medium containing Kanamycin (10 µg/ml) and incubated overnight at 37°C with shaking at 250 rpm.

Plasmids were extracted from the liquid cultures using QIAgen Plasmid Purification mini kit (Qiagen, USA) according to the manufacturer's instructions. However, the resuspension buffer was supplemented with 1 mg/ml of chicken egg white lysozyme (SIGMA, USA) prior to lysing the cells at 37°C for 15 minutes. 5 µl samples of the plasmids were digested with PstI and KpnI. The digestions were checked by gel electrophoresis on a 1.5% agarose gel (NuSieve™, FMC). The appearance of a DNA fragment of 837 bp, the same size as seen from the PCR amplification, indicated a positive clone. One positive clone was designated MOL1297.

Expression, secretion and functional analysis of the fusion protein

The clone MOL1297 (expressing *C. fimi cenA* CBD fused to the N-terminal of Termamyl™) was incubated for 20 hours in SB medium at 37°C with shaking at 250 rpm. 1 ml of cell-free supernatant was mixed with 200 µl of 10% Avicel™. The mixture was incubated for 1 hour at 0°C and then centrifuged for 5 min at 5000 x g. The pellet was resuspended in 100 µl of SDS-PAGE buffer, boiled at 95°C for 5 minutes, centrifuged at 5000 x g for 5 minutes, and 25 µl was loaded on a 4-20% Laemmli Tris-Glycine, SDS-PAGE NOVEX gel (Novex, USA). The samples were subjected to electrophoresis in an Xcell™ Mini-Cell (NOVEX, USA) as recommended by the manufacturer. All subsequent handling of gels including staining (Coomassie), destaining and drying, was performed as described by the manufacturer.

The appearance of a protein band of MW approx. 85 kDa indicated expression in *B. subtilis* of the CBD-Termamyl™ fusion.

The encoding sequence for the *C. fimi cenA* CBD-Termamyl hybrid is shown in SEQ ID No. 7 (in which lower case letters indicate the CBD-encoding part of the sequence). The corresponding amino acid sequence of the hybrid is shown in SEQ ID No. 8 (in which lower case letters indicate the CBD amino acid sequence).

EXAMPLE 4

This example describes the construction of fusion proteins (enzyme hybrid) from a lipase (Lipolase™; *Humicola*

lanuginosa lipase) and a CBD. A construction with an N-terminal CBD was chosen, since the N-terminal of the enzyme is far from the active site, whereas the C-terminal is in relatively close proximity to the active site.

pIVI450 construction (CBD-linker-lipase)

This construct was made in order to express a protein having the *Myceliophthora thermophila* cellulase CBD and linker at the N-terminal of Lipolase™.

A PCR fragment was created using the clone pA2C161 (DSM 9967) containing the *M. thermophila* cellulase gene as template, and the following oligomers as primers:

#8202
5' ACGTAGTGGCCACGCTAGGCGAGGTGGTGG 3'

#19672
5' CCACACTTCTCTTCCTTCCTC 3'

The PCR fragment was cut with BamHI and BalI, and cloned into pAHL which was also cut with BamHI and BalI just upstream of the presumed signal peptide processing site. The cloning was verified by sequencing (see SEQ ID No. 9).

Removing linker between CBD and lipase

This construct is made so that any linker of interest can be inserted between the CBD and the lipase in order to find an optimal linker.

An NheI site is introduced by the USE technique (Stratagene catalogue No. 200509) between the CBD and linker region in pIVI450, creating pIVI450+NheI site. pIVI450+NheI site is cut with XhoI and NheI, isolating the vector containing the

CBD part.

The plasmid pIVI392 is cut with XhoI and NheI, and the fragment containing the Lipolase™ gene (minus signal peptide encoding sequence) is isolated.

The DNA fragments are ligated, generating pIVI450 CBD-NheI site-Lipolase™ containing an NheI site between the CBD and the lipase gene. In this NheI site different linkers can be introduced.

Introduction of non-glycosylated linker

The protein expressed from the construct described here contains a construction of the type:
CBD-nonglycosylated linker-lipase.

The amino acid sequence of the linker is as follows:

NNNPQQGNPNQGGNNGGGNQGGGNGG

PCR is performed with the following primers:

#29315
5' GATCTAGCTAGCAACAATAACCCCCAGCAGGGCAACCCCAACCAGGGC GGGAACAACGGC 3'

#29316
5' GATCTAGCTAGCGCCGCCGTTGCCGCCGCCCTGGTTGCCGCCGCCGTT GTTCCCGCCCTG 3'

The PCR fragment is cut with NheI, the vector pIVI450 CBD-NheI-Lipolase™ is likewise cut with NheI, and the two fragments are ligated, creating:
pIVI450 CBD-Nonglycosylated linker-Lipolase™ (SEQ ID No.

10).

Introduction of *H. insolens* family 45 cellulase linker

The protein expressed from the construct described here contains a construction of the type:
CBD-glycosylated linker-lipase.

The amino acid sequence of the linker is as follows:

VQIPSSSTSSPVNQPTSTSTTSTSTTSSPPVQPTTPS

PCR is performed with the following primers:

#29313
5' GATACTGCTAGCGTCCAGATCCCCTCCAGC 3'

#29314
5' GATACTGCTAGCGCTGGGAGTCGTAGGCTG 3'

The PCR fragment is cut with NheI, the vector pIVI450 CBD-NheI-Lipolase™ is likewise cut with NheI, and the two fragments are ligated, creating:
pIVI450 CBD-*H. insolens* family 45 cellulase linker-Lipolase™ (SEQ ID No. 11).

EXAMPLE 5

This example concerns fusion proteins comprising a CBD linked to *Coprinus cinereus* peroxidase (CiP) or to a mutant thereof (mCiP842) (see, e.g., WO 95/10602).

Yeast expression system

The pJC106/YNG344 host/vector system was chosen as the standard expression system for all CiP experiments utilizing

yeast expression. Mutant mCiP842 contains the following amino acid substitutions relative to the parent CiP: V53A, E239G, Y272F, M242I. Constructions using this plasmid were performed with the same procedure as was used for the fusion of CBD to the wild type CiP gene.

Construction of the CBD-CiP fusion vector JC20A or JC20D: CiP signal seq.-*H. insolens* family 45 cellulase CBD- *H. insolens* family 45 cellulase linker-CiP or -mCiP842

The CBD-CiP fusion was constructed by amplifying four separate gene fragments using PCR. A) The CiP 5'-untranslated region and the CiP coding sequence from plasmid JC106 or mCiP842 encoding amino acids 1 to 22, B) the *H. insolens* family 45 cellulase CBD from plasmid pCaHj418 encoding amino acids 248-305, C) the *H. insolens* family 45 cellulase linker domain from plasmid pCaHj418 encoding amino acids 213-247, and D) the CiP coding sequence from plasmid JC106 or mCiP842 encoding amino acids 21 to 344.

The sequence of the *H. insolens* family 45 cellulase is disclosed in WO 91/17244.

Primers used in amplifications A through D were as follows:

Amplification A:

1. CiPpcrdwn: CCCCCTTCCCTGGCGAATTCCGCATGAGG
2. JC20.1: ACCTTGGGGTAGAGCGAGGGCACCGATG

Amplification B:

3. JC20.2: TGCACTGCTGAGAGGTGGGC
4. JC20.3: CAGGCACTGATGATACCAGT

Amplification C:

5. JC20.4: CCCTCCAGCAGCACCAGCTCT
6. JC20.5: TCCTCCAGGACCCTGACCGCTCGGAGTCGTAGGCTG

Amplification D:

7. JC20.6: TACGACTCCGAGCGGTCAGGGTCCTGGAGGAGGCGGG

8. YES2term: GGGAGGGCGTGAATGTAAG

Amplified products of reactions A) and B) were purified and phosphorylated using T4 polynucleotide kinase, ligated to one another for 15 min. at room temperature, and amplified with primers 1 and 4 to generate product AB. Amplified products of reactions C) and D) were purified and mixed, then PCR-amplified to generate product CD. Reaction products AB and CD were purified and phosphorylated using T4 polynucleotide kinase, ligated to one another for 15 min. at room temperature, and amplified with primers 1 and 8 to generate the final product. The resulting product was purified, mixed with plasmid JC106 which had the CiP gene removed by digestion with BamHI and XhoI. Plasmid JC20A contains the wild type CiP gene, whereas plasmid JC20D contains the peroxide-stable mutant mCiP842. Transformants were selected on minimal media lacking uridine.

Construction of the other CBD-CiP fusion vectors JC21, 22, 23

Other plasmids containing alternate linkers between the *H. insolens* family 45 cellulase CBD and CiP were constructed in essentially the same way as described for plasmid JC20A above, using PCR and overlap extension. The resulting plasmids encode fusion proteins with the following domain compositions:

JC21: CiP signal seq.-truncated *H. insolens* family 45 cellulase CBD-*H. insolens* family 45 cellulase linker-CiP

JC22: CiP signal seq.-*H. insolens* family 45 cellulase CBD-linker from the NifA gene of *Klebsiella pneumoniae*-CiP

JC23: CiP signal seq.-*H. insolens* family 45 cellulase CBD-

linker from the E. coli OmpA gene-CiP.

Scoring of transformants for peroxidase and cellulose-binding activity

Plate Assay: Yeast transformants were grown on minimal media plates containing 2% galactose (to induce the GAL1 yeast promoter driving CBD-CiP expression) that had been covered with a double filter layer consisting of cellulose acetate on top of nitrocellulose. After overnight growth, both filters were washed twice with 100 ml of 20 mM phosphate buffer, pH 7.0 for 5 minutes, after which no colony debris could be detected. Filters were then assayed for bound peroxidase activity by coating them with a 100 mM phosphate buffer, pH 7.0, containing 50 µg/ml of diamino-benzidine and 1 mM hydrogen peroxide. Bound peroxidase activity appears as a brown precipitate on the filter.

Liquid Assay: Liquid cultures of mutants demonstrating cellulose binding in the filter assay were grown overnight in minimal media containing 2% galactose. 20 µl samples of culture broth were mixed with Avicel crystalline cellulose (20 g/L) in 0.1 M phosphate buffer, pH 7, 0.01% Tween 20 in a total volume of 100 µl and incubated at 22°C for 10 minutes. The mixture was then centrifuged to pellet the insoluble cellulose fraction, and the supernatants were assayed for peroxidase activity using the standard CiP assay (see, e.g, WO 95/10602). Binding was scored as the % activity bound to the insoluble cellulose fraction based on the decrease in soluble activity.

High pH/thermal stability screening of CBD-CiP fusions

This screening process utilizes broth samples from yeast cultures grown in microtiter plates .The 96-well plate screen is performed by first growing yeast transformants of a pool

of mutants in 50 µL volumes of URA(-) medium, pH 6.0 in 96-well microtiter plates. Cultures are inoculated by dilution into medium and pipetting (robotic or manual autopipettor) into 96-well plates. These are placed in an incubator set at 30°C, 350 RPM and shaken for approximately 5 days. Plates are placed directly from the culture box onto the robotic system.

Both CiP and mCiP842 and the related fusion proteins were subjected to a combined pH - temperature - H_2O_2 stress test: After an initial activity assay, cultures are diluted to ca. 0.06 PODU/ml (see WO 95/10602 for definition of PODU) and incubated in 200 µM hydrogen peroxide, 100mM phosphate/borate buffer, pH10.5 at 50° C. After 0, 10, 20 and 30 minutes, samples are removed and residual activity is measured using the standard ABTS assay, pH 7.0. Improved mutants are those showing higher residual activity than CiP and are expressed as percent residual activity relative to the time 0 assay result.

Yeast expression plasmids designed to make five *H. insolens* family 45 cellulase CBD-CiP fusions were constructed and sequenced. The primary difference between the fusions is in the type of linker domain that connects the CBD to the CiP, as this was thought to be important for maximizing the binding of the CBD to cellulosic substrates.

All the constructs encode a fusion of four discrete domains: CiP signal sequence-*H. insolens* family 45 cellulase CBD-linker-CiP. Plasmid JC20A is a CBD-CiP fusion to the wild type CiP, while plasmid JC20D is a fusion to the stable mutant mCiP842 containing the amino acid substitutions V53A, E239G, M242I and Y272F. Both JC20 constructs contain the natural *H. insolens* family 45 cellulase linker domain.

Plasmid JC21 encodes a fusion protein identical to the JC20 product with the exception that it contains a truncated linker lacking residues 7 to 23 of the *H. insolens* family 45 cellulase linker. Plasmid JC22 has the *H. insolens* family 45 cellulase linker domain replaced with a 12 residue proline-rich linker from the outer membrane protein of *E. coli* (from the OmpA gene). The final plasmid, JC23, contains a fourth linker (called a Q linker) derived from the NifA gene of *Klebsiella pneumoniae*. This linker, 14 amino acids in length, contains 3 glutamine residues (hence the name Q linker) as well as 3 arginine residues, giving it a positive charge at neutral pH.

These JC20-series plasmids were transformed into *S. cerevisae* for expression and testing. After transformation, yeast colonies were grown on selective plates covered with a double filter layer: cellulose acetate filters on top of nitrocellulose. Wild type CiP secreted from yeast JC106 and the stable mutant mCiP842 pass through the cellulose acetate, then binds to the nitrocellulose where it can be visualized using diaminobenzidine (DAB) and H_2O_2. The cellulose acetate filter does not bind any wild-type or mCiP842 peroxidase. In contrast, the N-terminal CBD-CiP fusions encoded by plasmids JC20A, JC20D, JC21, JC22, and JC23 are all detectable on both filters using the DAB assay, indicating that the fusion proteins have both peroxidase and cellulose-binding activities. Visual inspection of filters suggests that the NifA linker may improve binding slightly over the others, although the difference is marginal. In all cases the peroxidase activity bound to the cellulose acetate filter remains bound even after washing extensively with buffer at pH 7. The activity bound to the lower nitrocellulose filter suggests that binding of the CBD-CiP may be incomplete, or the cellulose filter gets saturated, allowing some of the

fusion protein to pass through to the lower filter, or that some percentage of the fusion protein gets truncated to include only the peroxidase domain.

Sequence identifiers herein corresponding to the constructs are as as indicated below. Abbreviations are as follows:

EGV: *Humicola insolens* family 45 endoglucanase (cellulase)
CiPss: CiP signal sequence
CiP842: CiP mutant/variant mCiP842;

SEQ ID No. 12: Nucleotide sequence of the CiPss(+ 2 amino acids)-EGV CBD-EGV linker-CiP fusion in JC20.A;

SEQ ID No.13: Nucleotide sequence of the CiPss(+ 2 amino acids)-EGV CBD-EGV linker-CiP842 fusion in JC20.D1;

SEQ ID NO. 14: Nucleotide sequence of the CiPss(+ 2 amino acids)-EGV CBD-truncated EGV linker-CiP fusion in JC21;

SEQ ID No. 15: Nucleotide sequence of the CiPss(+ 2 amino acids)-EGV CBD-*E. coli* OmpA linker-CiP fusion in JC22;

SEQ ID No. 16: Nucleotide sequence of the CiPss(+ 2 amino acids)-EGV CBD-NifA linker- CiP fusion in JC23.

EXAMPLE 6

This example concerns fusion proteins comprising a CBD linked to *Myceliophthora thermophila* laccase (MtL) (MtL is described in, e.g., WO 95/33836).

Construction of the N-terminal MtL-CBD fusion pJC24

A DNA fragment containing the *Coprinus cinereus* peroxidase (CiP) signal sequence (22 amino acids), the *H. insolens* family 45 cellulase CBD (37 amino acids) and a NifA linker domain from *Klebsiella pneumoniae* (14 amino acids) was PCR-amplified using two specific primers to plasmid pJC23.

primer name	sequence
CiPpcrdwn:	CTGGGGTAATTAATCAGCGAAGCGATG
JC24.1	AGCGCGTGGACGTTCGATGC

PCR amplification was performed using Pwo polymerase (Boehringer Mannheim) using the supplied buffer according to the manufacturer's instructions. The reaction was initiated after 3 min. at 96°C by addition of the polymerase, and allowed to cycle 30 times with 30 sec at 96°C, 30 sec at 60°C and 2 min at 72°C. A second PCR fragment encoding the mature MtL peptide lacking both the signal peptide and propeptide (residues 48-620) was PCR amplified from a cDNA clone of the *Myceliophthora* laccase contained in plasmid pJRoC30. PCR amplification was performed using the same conditions as described above and the following primer pair:

primer name	sequence
JC24.2	CAGCAGAGCTGCAACACCCCCAG
YES2term	GGGGAGGGCGTGAATGTAAG

Following amplification, both DNA fragments were purified using the QiaQuick™ Spin purification kit (Qiagen, Inc.) according to the manufacturer's recommendations. The two DNA fragments were then ligated together and a portion of the ligation mix used as a template for PCR amplification using the CiPpcrdwn and YES2term primers under the same conditions as described above. The resulting 2.3 kb chimeric DNA fragment was gel-purified, cut with BamHI and NotI restriction enzymes, and ligated into the vector

backbone of plasmid pJC106 to obtain plasmid pJC24.

Construction of th C-terminal MtL-CBD fusion pJC25

A PCR fragment encoding the entire MtL peptide (residues 1-620) and 232 bp of upstream sequence was amplified from plasmid pJRoC30 using the following primer pair:

primer name	sequence
CiPpcrdwn:	CTGGGGTAATTAATCAGCGAAGCGATG
JC25.2	CGCCTTGACCAGCCACTCGCCCTCCTCG

A second DNA fragment encoding the *H. insolens* family 45 cellulase linker domain (35 amino acids), the *H. insolens* family 45 cellulase CBD (37 amino acids) and 20 bp of 3' non-coding sequence was amplified from the *H. insolens* family 45 cellulase plasmid pCaHj418 using the following primer pair:

primer name	sequence
JC20.4	CCCTCCAGCAGCACCAGCTCTC
JC25.1NotI	ATAAGAATGCGGCCGCCTACAGGCACTGATGGTACCAGT

The two DNA fragments were ligated briefly and the full-length 2.3 kb fusion product was amplified as described above, using the primers CiPpcrdwn and JC25.1NotI. This final PCR product was cloned into plasmid pJC106 to obtain plasmid pJC25.

Construction of the C-terminal MtL-CBD fusion pJC26

Plasmid pJC26 was constructed in exactly the same manner as pJC25, except that primer ML-ct was substituted for primer JC25.1 and resulted in a truncated product of the MtL gene lacking the final 17 codons.

primer name	sequence

ML-ct CAGCAGAGCTGCAACACC

Sequence identifiers herein corresponding to the constructs are as as indicated below. Abbreviations are as follows:

EGV: *Humicola insolens* family 45 endoglucanase (cellulase)
CiPss: CiP signal sequence
MtLss: MtL signal sequence

SEQ ID No. 17: Nucleotide sequence of the CiPss(+ 2 amino acids)-EGV CBD-NifA linker-MtL fusion in pJC24;

SEQ ID No. 18: Nucleotide sequence of the MtLss-MtL propeptide-MtL-EGV linker-EGV CBD fusion in pJC25;

SEQ ID No. 19: Nucleotide sequence of the MtLss-MtL propeptide-MtL (minus 17 amino acids)-EGV linker-EGV CBD fusion in pJC26. The codons corresponding to the 17 amino acids in question are shown in bold in SEQ ID No. 18.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: NOVO NORDISK A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: +45 44 44 88 88
(H) TELEFAX: +45 44 49 32 56

(ii) TITLE OF INVENTION: PROCESS FOR REMOVAL OR BLEACHING OF SOILING OR STAINS FROM CELLULOSIC FABRIC

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2253 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATGAAACAAC AAAAACGGCT TTACGCCCGA TTGCTGACGC TGTTATTTGC GCTCATCTTC 60

TTGCTGCCTC ATTCTGCAGC AGCGGCGGCA AATCTTAATG GGACGCTGAT GCAGTATTTT 120

GAATGGTACA TGCCCAATGA CGGCCAACAT TGGAAGCGTT TGCAAAACGA CTCGGCATAT 180

TTGGCTGAAC ACGGTATTAC TGCCGTCTGG ATTCCCCCGG CATATAAGGG AACGAGCCAA 240

GCGGATGTGG GCTACGGTGC TTACGACCTT TATGATTTAG GGGAGTTTCA TCAAAAAGGG 300

ACGGTTCGGA CAAAGTACGG CACAAAAGGA GAGCTGCAAT CTGCGATCAA AAGTCTTCAT 360

TCCCGCGACA TTAACGTTTA CGGGGATGTG GTCATCAACC ACAAAGGCGG CGCTGATGCG 420

ACCGAAGATG TAACCGCGGT TGAAGTCGAT CCCGCTGACC GCAACCGCGT AATCTCAGGA 480

GAACACCTAA TTAAAGCCTG GACACATTTT CATTTTCCGG GGGCCGGCAG CACATACAGC 540

GATTTTAAAT GGCATTGGTA CCATTTTGAC GGAACCGATT GGGACGAGTC CCGAAAGCTG 600

AACCGCATCT ATAAGTTTCA AGGAAAGGCT TGGGATTGGG AAGTTTCCAA TGAAAACGGC 660

AACTATGATT ATTTGATGTA TGCCGACATC GATTATGACC ATCCTGATGT CGCAGCAGAA 720

ATTAAGAGAT GGGGCACTTG GTATGCCAAT GAACTGCAAT TGGACGGAAA CCGTCTTGAT 780

GCTGTCAAAC ACATTAAATT TTCTTTTTTG CGGGATTGGG TTAATCATGT CAGGGAAAAA 840

ACGGGGAAGG AAATGTTTAC GGTAGCTGAA TATTGGCAGA ATGACTTGGG CGCGCTGGAA 900

AACTATTTGA ACAAAACAAA TTTTAATCAT TCAGTGTTTG ACGTGCCGCT TCATTATCAG 960

TTCCATGCTG CATCGACACA GGGAGGCGGC TATGATATGA GGAAATTGCT GAACGGTACG 1020

GTCGTTTCCA AGCATCCGTT GAAATCGGTT ACATTTGTCG ATAACCATGA TACACAGCCG 1080

GGGCAATCGC TTGAGTCGAC TGTCCAAACA TGGTTTAAGC CGCTTGCTTA CGCTTTTATT 1140

CTCACAAGGG AATCTGGATA CCCTCAGGTT TTCTACGGGG ATATGTACGG GACGAAAGGA 1200

GACTCCCAGC GCGAAATTCC TGCCTTGAAA CACAAAATTG AACCGATCTT AAAAGCGAGA 1260

AAACAGTATG CGTACGGAGC ACAGCATGAT TATTTCGACC ACCATGACAT TGTCGGCTGG 1320

ACAAGGGAAG GCGACAGCTC GGTTGCAAAT TCAGGTTTGG CGGCATTAAT AACAGACGGA 1380

CCCGGTGGGG CAAAGCGAAT GTATGTCGGC CGGCAAAACG CCGGTGAGAC ATGGCATGAC 1440

ATTACCGGAA ACCGTTCGGA GCCGGTTGTC ATCAATTCGG AAGGCTGGGG AGAGTTTCAC 1500

GTAAACGGCG GATCCGTTTC AATTTATGTT CAAAGATCTG GCGGACCTGG AACGCCAAAT 1560

AATGGCAGAG GAATTGGTTA TATTGAAAAT GGTAATACCG TAACTTACAG CAATATAGAT 1620

TTTGGTAGTG GTGCAACAGG GTTCTCTGCA ACTGTTGCAA CGGAGGTTAA TACCTCAATT 1680

CAAATCCGTT CTGACAGTCC TACCGGAACT CTACTTGGTA CCTTATATGT AAGTTCTACC 1740

GGCAGCTGGA ATACATATCA ACCGTATCTA CAAACATCAG CAAAATTACC GGCGTTCATG 1800

ATATTGTATT GGTATTCTCA GGTCCAGTCA ATGTGGACAA CTTCATATTT AGCAGAAGTT 1860

CACCAGTGCC TGCACCTGGT GATAACACAA GAGACGCATA TTCTATCATT CAGGCCGAGG 1920

ATTATGACAG CAGTTATGGT CCCAACCTTC AAATCTTTAG CTTACCAGGT GGTGGCAGCG 1980

CTTGGCTATA TTGAAAATGG TTATTCCACT ACCTATAAAA ATATTGATTT TGGTGACGGC 2040

GCAACGTCCG TAACAGCAAG AGTAGCTACC CAGAATGCTA CTACCATTCA GGTAAGATTG 2100

GGAAGTCCAT CGGGTACATT ACTTGGAACA ATTTACGTGG GGTCCACAGG AAGCTTTGAT 2160

ACTTATAGGG ATGTATCCGC TACCATTAGT AATACTGCGG GTGTAAAAGA TATTGTTCTT 2220

GTATTCTCAG GTCCTGTTAA TGTTGACTGG TAG 2253

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 750 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Lys Gln Gln Lys Arg Leu Tyr Ala Arg Leu Leu Thr Leu Leu Phe
1 5 10 15

Ala Leu Ile Phe Leu Leu Pro His Ser Ala Ala Ala Ala Ala Asn Leu
20 25 30

Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro Asn Asp Gly
35 40 45

Gln His Trp Lys Arg Leu Gln Asn Asp Ser Ala Tyr Leu Ala Glu His
50 55 60

Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Thr Ser Gln
65 70 75 80

Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu Gly Glu Phe
85 90 95

His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Gly Glu Leu
100 105 110

Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn Val Tyr Gly
115 120 125

Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr Glu Asp Val
130 135 140

Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val Ile Ser Gly
145 150 155 160

Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro Gly Ala Gly
165 170 175

Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly Thr
180 185 190

Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys Phe Gln Gly
195 200 205

Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn Tyr Asp Tyr
210 215 220

Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val Ala Ala Glu
225 230 235 240

Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln Leu Asp Gly
245 250 255

Asn Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe Leu Arg Asp
260 265 270

Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met Phe Thr Val
275 280 285

Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn Tyr Leu Asn
290 295 300

Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu His Tyr Gln
305 310 315 320

Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met Arg Lys Leu
325 330 335

Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser Val Thr Phe
340 345 350

Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu Ser Thr Val
355 360 365

Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu Thr Arg Glu
370 375 380

Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly Thr Lys Gly
385 390 395 400

Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile Glu Pro Ile
405 410 415

Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His Asp Tyr Phe
420 425 430

Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp Ser Ser Val
435 440 445

Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro Gly Gly Ala
450 455 460

Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr Trp His Asp
465 470 475 480

Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser Glu Gly Trp
485 490 495

Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr Val Gln Arg
500 505 510

Ser Gly Gly Pro Gly Thr Pro Asn Asn Gly Arg Gly Ile Gly Tyr Ile
515 520 525

Glu Asn Gly Asn Thr Val Thr Tyr Ser Asn Ile Asp Phe Gly Ser Gly
530 535 540

Ala Thr Gly Phe Ser Ala Thr Val Ala Thr Glu Val Asn Thr Ser Ile
545 550 555 560

Gln Ile Arg Ser Asp Ser Pro Thr Gly Thr Leu Leu Gly Thr Leu Tyr
565 570 575

Val Ser Ser Thr Gly Ser Trp Asn Thr Tyr Gln Pro Tyr Leu Gln Thr
580 585 590

Ser Ala Lys Leu Pro Ala Phe Met Ile Leu Tyr Trp Tyr Ser Gln Val
595 600 605

Gln Ser Met Trp Thr Thr Ser Tyr Leu Ala Glu Val His Gln Cys Leu
610 615 620

His Leu Val Ile Thr Gln Glu Thr His Ile Leu Ser Phe Arg Pro Arg
625 630 635 640

Ile Met Thr Ala Val Met Val Pro Thr Phe Lys Ser Leu Ala Tyr Gln
645 650 655

Val Val Ala Ala Leu Gly Tyr Ile Glu Asn Gly Tyr Ser Thr Thr Tyr
660 665 670

Lys Asn Ile Asp Phe Gly Asp Gly Ala Thr Ser Val Thr Ala Arg Val
675 680 685

Ala Thr Gln Asn Ala Thr Thr Ile Gln Val Arg Leu Gly Ser Pro Ser
690 695 700

Gly Thr Leu Leu Gly Thr Ile Tyr Val Gly Ser Thr Gly Ser Phe Asp
705 710 715 720

Thr Tyr Arg Asp Val Ser Ala Thr Ile Ser Asn Thr Ala Gly Val Lys
725 730 735

Asp Ile Val Leu Val Phe Ser Gly Pro Val Asn Val Asp Trp
740 745 750

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1203 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATGAAAAAGA TAACTACTAT TTTTGTCGTA TTGCTTATGA CAGTGGCGTT GTTCAGTATA 60

GGAAACACGA CTGCTGCTGA TAATGATTCA GTTGTAGAAG AACATGGGCA ATTAAGTATT 120

AGTAACGGTG AATTAGTCAA TGAACGAGGC GAACAAGTTC AGTTAAAAGG GATGAGTTCC 180

CATGGTTTGC AATGGTACGG TCAATTTGTA AACTATGAAA GTATGAAATG GCTAAGAGAT 240

GATTGGGGAA TAAATGTATT CCGAGCAGCA ATGTATACCT CTTCAGGAGG ATATATTGAT 300

GATCCATCAG TAAAGGAAAA AGTAAAAGAG GCTGTTGAAG CTGCGATAGA CCTTGATATA 360

TATGTGATCA TTGATTGGCA TATCCTTTCA GACAATGACC CAAATATATA TAAAGAAGAA 420

GCGAAGGATT TCTTTGATGA AATGTCAGAG TTGTATGGAG ACTATCCGAA TGTGATATAC 480

GAAATTGCAA ATGAACCGAA TGGTAGTGAT GTTACGTGGG GCAATCAAAT AAAACCGTAT 540

GCAGAGGAAG TCATTCCGAT TATTCGTAAC AATGACCCTA ATAACATTAT TATTGTAGGT 600

ACAGGTACAT GGAGTCAGGA TGTCCATCAT GCAGCTGATA ATCAGCTTGC AGATCCTAAC 660

GTCATGTATG CATTTCATTT TTATGCAGGG ACACATGGTC AAAATTTACG AGACCAAGTA 720

GATTATGCAT TAGATCAAGG AGCAGCGATA TTTGTTAGTG AATGGGGAAC AAGTGCAGCT 780

ACAGGTGATG GTGGCGTGTT TTTAGATGAA GCACAAGTGT GGATTGACTT TATGGATGAA 840

AGAAATTTAA GCTGGGCCAA CTGGTCTCTA ACGCATAAAG ATGAGTCATC TGCAGCGTTA 900

ATGCCAGGTG CAAATCCAAC TGGTGGTTGG ACAGAGGCTG AACTATCTCC ATCTGGTACA 960

TTTGTGAGGG AAAAAATAAG AGAATCAGCA TCTATTCCGC CAAGCGATCC AACACCGCCA 1020

TCTGATCCAG GAGAACCGGA TCCAACGCCC CCAAGTGATC CAGGAGAGTA TCCAGCATGG 1080

GATCCAAATC AAATTTACAC AAATGAAATT GTGTACCATA ACGGCCAGCT ATGGCAAGCA 1140

AAATGGTGGA CACAAAATCA AGAGCCAGGT GACCCGTACG GTCCGTGGGA ACCACTCAAT 1200

TAA 1203

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 400 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Lys Lys Ile Thr Thr Ile Phe Val Val Leu Leu Met Thr Val Ala
1 5 10 15

Leu Phe Ser Ile Gly Asn Thr Thr Ala Ala Asp Asn Asp Ser Val Val
20 25 30

Glu Glu His Gly Gln Leu Ser Ile Ser Asn Gly Glu Leu Val Asn Glu
35 40 45

Arg Gly Glu Gln Val Gln Leu Lys Gly Met Ser Ser His Gly Leu Gln
50 55 60

Trp Tyr Gly Gln Phe Val Asn Tyr Glu Ser Met Lys Trp Leu Arg Asp
65 70 75 80

Asp Trp Gly Ile Asn Val Phe Arg Ala Ala Met Tyr Thr Ser Ser Gly
85 90 95

Gly Tyr Ile Asp Asp Pro Ser Val Lys Glu Lys Val Lys Glu Ala Val
100 105 110

Glu Ala Ala Ile Asp Leu Asp Ile Tyr Val Ile Ile Asp Trp His Ile
115 120 125

Leu Ser Asp Asn Asp Pro Asn Ile Tyr Lys Glu Glu Ala Lys Asp Phe
130 135 140

Phe Asp Glu Met Ser Glu Leu Tyr Gly Asp Tyr Pro Asn Val Ile Tyr
145 150 155 160

Glu Ile Ala Asn Glu Pro Asn Gly Ser Asp Val Thr Trp Gly Asn Gln
165 170 175

Ile Lys Pro Tyr Ala Glu Glu Val Ile Pro Ile Ile Arg Asn Asn Asp
180 185 190

Pro Asn Asn Ile Ile Ile Val Gly Thr Gly Thr Trp Ser Gln Asp Val
195 200 205

His His Ala Ala Asp Asn Gln Leu Ala Asp Pro Asn Val Met Tyr Ala
210 215 220

Phe His Phe Tyr Ala Gly Thr His Gly Gln Asn Leu Arg Asp Gln Val
225 230 235 240

Asp Tyr Ala Leu Asp Gln Gly Ala Ala Ile Phe Val Ser Glu Trp Gly
245 250 255

Thr Ser Ala Ala Thr Gly Asp Gly Gly Val Phe Leu Asp Glu Ala Gln
260 265 270

Val Trp Ile Asp Phe Met Asp Glu Arg Asn Leu Ser Trp Ala Asn Trp
275 280 285

Ser Leu Thr His Lys Asp Glu Ser Ser Ala Ala Leu Met Pro Gly Ala
290 295 300

Asn Pro Thr Gly Gly Trp Thr Glu Ala Glu Leu Ser Pro Ser Gly Thr
305 310 315 320

Phe Val Arg Glu Lys Ile Arg Glu Ser Ala Ser Ile Pro Pro Ser Asp
325 330 335

Pro Thr Pro Pro Ser Asp Pro Gly Glu Pro Asp Pro Thr Pro Pro Ser
340 345 350

Asp Pro Gly Glu Tyr Pro Ala Trp Asp Pro Asn Gln Ile Tyr Thr Asn
355 360 365

Glu Ile Val Tyr His Asn Gly Gln Leu Trp Gln Ala Lys Trp Trp Thr
370 375 380

Gln Asn Gln Glu Pro Gly Asp Pro Tyr Gly Pro Trp Glu Pro Leu Asn
385 390 395 400

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1683 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ATGAAACAAC AAAAACGGCT TTACGCCCGA TTGCTGACGC TGTTATTTGC GCTCATCTTC 60

TTGCTGCCTC ATTCTGCAGC AGCGGCGGCA AATCTTAATG GGACGCTGAT GCAGTATTTT 120

GAATGGTACA TGCCCAATGA CGGCCAACAT TGGAAGCGTT TGCAAAACGA CTCGGCATAT 180

TTGGCTGAAC ACGGTATTAC TGCCGTCTGG ATTCCCCCGG CATATAAGGG AACGAGCCAA 240

GCGGATGTGG GCTACGGTGC TTACGACCTT TATGATTTAG GGGAGTTTCA TCAAAAAGGG 300

ACGGTTCGGA CAAAGTACGG CACAAAAGGA GAGCTGCAAT CTGCGATCAA AAGTCTTCAT 360

TCCCGCGACA TTAACGTTTA CGGGGATGTG GTCATCAACC ACAAAGGCGG CGCTGATGCG 420

ACCGAAGATG TAACCGCGGT TGAAGTCGAT CCCGCTGACC GCAACCGCGT AATCTCAGGA 480

GAACACCTAA TTAAAGCCTG GACACATTTT CATTTTCCGG GGGCCGGCAG CACATACAGC 540

GATTTTAAAT GGCATTGGTA CCATTTTGAC GGAACCGATT GGGACGAGTC CCGAAAGCTG 600

AACCGCATCT ATAAGTTTCA AGGAAAGGCT TGGGATTGGG AAGTTTCCAA TGAAAACGGC 660

AACTATGATT ATTTGATGTA TGCCGACATC GATTATGACC ATCCTGATGT CGCAGCAGAA 720

ATTAAGAGAT GGGGCACTTG GTATGCCAAT GAACTGCAAT TGGACGGAAA CCGTCTTGAT 780

GCTGTCAAAC ACATTAAATT TTCTTTTTTG CGGGATTGGG TTAATCATGT CAGGGAAAAA 840

ACGGGGAAGG AAATGTTTAC GGTAGCTGAA TATTGGCAGA ATGACTTGGG CGCGCTGGAA 900

AACTATTTGA ACAAAACAAA TTTTAATCAT TCAGTGTTTG ACGTGCCGCT TCATTATCAG 960

TTCCATGCTG CATCGACACA GGGAGGCGGC TATGATATGA GGAAATTGCT GAACGGTACG 1020

GTCGTTTCCA AGCATCCGTT GAAATCGGTT ACATTTGTCG ATAACCATGA TACACAGCCG 1080

GGGCAATCGC TTGAGTCGAC TGTCCAAACA TGGTTTAAGC CGCTTGCTTA CGCTTTTATT 1140

CTCACAAGGG AATCTGGATA CCCTCAGGTT TTCTACGGGG ATATGTACGG GACGAAAGGA 1200

GACTCCCAGC GCGAAATTCC TGCCTTGAAA CACAAAATTG AACCGATCTT AAAAGCGAGA 1260

AAACAGTATG CGTACGGAGC ACAGCATGAT TATTTCGACC ACCATGACAT TGTCGGCTGG 1320

ACAAGGGAAG GCGACAGCTC GGTTGCAAAT TCAGGTTTGG CGGCATTAAT AACAGACGGA 1380

CCCGGTGGGG CAAAGCGAAT GTATGTCGGC CGGCAAAACG CCGGTGAGAC ATGGCATGAC 1440

ATTACCGGAA ACCGTTCGGA GCCGGTTGTC ATCAATTCGG AAGGCTGGGG AGAGTTTCAC 1500

GTAAACGGCG GATCCGTTTC AATTTATGTT CAAAGATCTC CTGGAGAGTA TCCAGCATGG 1560

GATCCAAATC AAATTTACAC AAATGAAATT GTGTACCATA ACGGCCAGCT ATGGCAAGCA 1620

AAATGGTGGA CACAAAATCA AGAGCCAGGT GACCCGTACG GTCCGTGGGA ACCACTCAAT 1680

TAA 1683

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 560 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Lys Gln Gln Lys Arg Leu Tyr Ala Arg Leu Leu Thr Leu Leu Phe
1 5 10 15

Ala Leu Ile Phe Leu Leu Pro His Ser Ala Ala Ala Ala Ala Asn Leu
20 25 30

Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro Asn Asp Gly
35 40 45

Gln His Trp Lys Arg Leu Gln Asn Asp Ser Ala Tyr Leu Ala Glu His
50 55 60

Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Thr Ser Gln
65 70 75 80

Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu Gly Glu Phe
85 90 95

His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Gly Glu Leu
100 105 110

Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn Val Tyr Gly
115 120 125

Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr Glu Asp Val
130 135 140

Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val Ile Ser Gly
145 150 155 160

Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro Gly Ala Gly
165 170 175

Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly Thr
180 185 190

Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys Phe Gln Gly

195 200 205

Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn Tyr Asp Tyr
210 215 220

Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val Ala Ala Glu
225 230 235 240

Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln Leu Asp Gly
245 250 255

Asn Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe Leu Arg Asp
260 265 270

Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met Phe Thr Val
275 280 285

Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn Tyr Leu Asn
290 295 300

Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu His Tyr Gln
305 310 315 320

Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met Arg Lys Leu
325 330 335

Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser Val Thr Phe
340 345 350

Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu Ser Thr Val
355 360 365

Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu Thr Arg Glu
370 375 380

Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly Thr Lys Gly
385 390 395 400

Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile Glu Pro Ile
405 410 415

Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His Asp Tyr Phe

420 425 430

Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp Ser Ser Val
435 440 445

Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro Gly Gly Ala
450 455 460

Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr Trp His Asp
465 470 475 480

Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser Glu Gly Trp
485 490 495

Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr Val Gln Arg
500 505 510

Ser Pro Gly Glu Tyr Pro Ala Trp Asp Pro Asn Gln Ile Tyr Thr Asn
515 520 525

Glu Ile Val Tyr His Asn Gly Gln Leu Trp Gln Ala Lys Trp Trp Thr
530 535 540

Gln Asn Gln Glu Pro Gly Asp Pro Tyr Gly Pro Trp Glu Pro Leu Asn
545 550 555 560

SEQ ID No. 7:

ATGAAACAACAAAAACGGCTTTACGCCCGATTGCTGACGCTGTTATTTGCGCTCATCTTCT
TGCTGCCTCATTCTGCAGC
AGCGGCGGCAAATCTTAATgctcccggctgccgcgtcgactacgccgtcaccaaccagtgg
cccggcggcttcggcgcca
acgtcacgatcaccaacctcggcgaccccgtctcgtcgtggaagctcgactggacctacac
cgcaggccagcggatccag
cagctgtggaacggcaccgcgtcgaccaacggcggccaggtctccgtcaccagcctgccct
ggaacggcagcatcccgac
cggcggcacggcgtcgttcgggttcaacggctcgtgggccgggtccaacccgacgccggcg
tcgttctcgctcaacggca
ccacgtgcactggtacagttcctacaactagtcctacacgtGCAAATCTTAATGGGACGCT
GATGCAGTATTTTGAATGG
TACATGCCCAATGACGGCCAACATTGGAGGCGTTTGCAAAACGACTCGGCATATTTGGCTG
AACACGGTATTACTGCCGT
CTGGATTCCCCCGGCATATAAGGGAACGAGCCAAGCGGATGTGGGCTACGGTGCTTACGAC

CTTTATGATTTAGGGGAGT
TTCATCAAAAAGGGACGGTTCGGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGAT
CAAAAGTCTTCATTCCCGC
GACATTAACGTTTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCGACCGAAG
ATGTAACCGCGGTTGAAGT
CGATCCCGCTGACCGCAACCGCGTAATTTCAGGAGAACACCTAATTAAAGCCTGGACACAT
TTTCATTTTCCGGGGCGCG
GCAGCACATACAGCGATTTTAAATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGA
GTCCCGAAAGCTGAACCGC
ATCTATAAGTTTCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGCAACTATG
ATTATTTGATGTATGCCGA
CATCGATTATGACCATCCTGATGTCGCAGCAGAAATTAAGAGATGGGGCACTTGGTATGCC
AATGAACTGCAATTGGACG
GTTTCCGTCTTGATGCTGTCAAACACATTAAATTTTCTTTTTTGCGGGATTGGGTTAATCA
TGTCAGGGAAAAAACGGGG
AAGGAAATGTTTACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAAAACTATT
TGAACAAAACAAATTTTAA
TCATTCAGTGTTTGACGTGCCGCTTCATTATCAGTTCCATGCTGCATCGACACAGGGAGGC
GGCTATGATATGAGGAAAT
TGCTGAACGGTACGGTCGTTTCCAAGCATCCGTTGAAATCGGTTACATTTGTCGATAACCA
TGATACACAGCCGGGGCAA
TCGCTTGAGTCGACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATTCTCACAA
GGGAATCTGGATACCCTCA
GGTTTTCTACGGGGATATGTACGGGACGAAAGGAGACTCCCAGCGCGAAATTCCTGCCTTG
AAACACAAAATTGAACCGA
TCTTAAAAGCGAGAAAACAGTATGCGTACGGAGCACAGCATGATTATTTCGACCACCATGA
CATTGTCGGCTGGACAAGG
GAAGGCGACAGCTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGACCCGGTG
GGGCAAAGCGAATGTATGT
CGGCCGGCAAAACGCCGGTGAGACATGGCATGACATTACCGGAAACCGTTCGGAGCCGGTT
GTCATCAATTCGGAAGGCT
GGGGAGAGTTTCACGTAAACGGCGGGTCGGTTTCAATTTATGTTCAAAGATAG

SEQ ID No. 8:

MKQQKRLYARLLTLLFALIFLLPHSAAAAanlnapgcrvdyavtnqwpgggfganvtitnlg
dpvsswkldwtytaggriq
qlwngtastnggqvsvtslpwngsiptggtasfgfngswagsnptpasfslngttctgtvp
ttsptrANLNGTLMQYFEW
YMPNDGQHWRRLQNDSAYLAEHGITAVWIPPAYKGTSQADVGYGAYDLYDLGEFHQKGTVR
TKYGTKGELQSAIKSLHSR
DINVYGDVVINHKGGADATEDVTAVEVDPADRNRVISGEHLIKAWTHFHFPGRGSTYSDFK
WHWYHFDGTDWDESRKLNR
IYKFQGKAWDWEVSNENGNYDYLMYADIDYDHPDVAAEIKRWGTWYANELQLDGFRLDAVK
HIKFSFLRDWVNHVREKTG
KEMFTVAEYWQNDLGALENYLNKTNFNHSVFDVPLHYQFHAASTQGGGYDMRKLLNGTVVS
KHPLKSVTFVDNHDTQPGQ

SLESTVQTWFKPLAYAFILTRESGYPQVFYGDMYGTKGDSQREIPALKHKIEPILKARKQY
AYGAQHDYFDHHDIVGWTR
EGDSSVANSGLAALITDGPGGAKRMYVGRQNAGETWHDITGNRSEPVVINSEGWGEFHVNG
GSVSIYVQRZ

SEQ ID No. 9:

GCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCAC
GACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCA
CTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGT
GAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGC
CTGCAGGTCGACGCATTCCGAATACGAGGCCTGATTAATGATTACATACGCCTCCGGGTAG
TAGACCGAGCAGCCGAGCCAGTTCAGCGCCTAAAACGCCTTATACAATTAAGCAGTTAAAG
AAGTTAGAATCTACGCTTAAAAAGCTACTTAAAAATCGATCTCGCAGTCCCGATTCGCCTA
TCAAAACCAGTTTAAATCAACTGATTAAAGGTGCCGAACGAGCTATAAATGATATAACAAT
ATTAAAGCATTAATTAGAGCAATATCAGGCCGCGCACGAAAGGCAACTTAAAAAGCGAAAG
CGCTCTACTAAACAGATTACTTTTGAAAAAGGCACATCAGTATTTAAAGCCCGAATCCTTA
TTAAGCGCCGAAATCAGGCAGATAAAGCCATACAGGCAGATAGACCTCTACCTATTAAATC
GGCTTCTAGGCGCGCTCCATCTAAATGTTCTGGCTGTGGTGTACAGGGGCATAAAATTACG
CACTACCCGAATCGATAGAACTACTCATTTTTATATAGAAGTCAGAATTCATAGTGTTTTG
ATCATTTTAAATTTTTATATGGCGGGTGGTGGGCAACTCGCTTGCGCGGGCAACTCGCTTA
CCGATTACGTTAGGGCTGATATTTACGTGAAAATCGTCAAGGGATGCAAGACCAAAGTAGT
AAAACCCCGGAAGTCAACAGCATCCAAGCCCAAGTCCTTCACGGAGAAACCCCAGCGTCCA
CATCACGAGCGAAGGACCACCTCTAGGCATCGGACGCACCATCCAATTAGAAGCAGCAAAG
CGAAACAGCCCAAGAAAAAGGTCGGCCCGTCGGCCTTTTCTGCAACGCTGATCACGGGCAG
CGATCCAACCAACACCCTCCAGAGTGACTAGGGGCGGAAATTTAAAGGGATTAATTTCCAC
TCAACCACAAATCACAGTCGTCCCCGGTATTGTCCTGCAGAATGCAATTTAAACTCTTCTG
CGAATCGCTTGGATTCCCCGCCCCTAGTCGTAGAGCTTAAAGTATGTCCCTTGTCGATGCG
ATGATACACAACATATAAATACTAGCAAGGGATGCCATGCTTGGAGGATAGCAACCGACAA
CATCACATCAAGCTCTCCCTTCTCTGAACAATAAACCCCACAGGGGGGATCCACTAGTAAC
GGCCGCCAGTGTGCTGGAAAGCGACTTGAAACGCCCCAAATGAAGTCCTCCATCCTCGCCA
GCGTCTTCGCCACGGGCGCCGTGGCTCAAAGTGGTCCGTGGCAGCAATGTGGTGGCATCGG
ATGGCAAGGATCGACCGACTGTGTGTCGGGCTACCACTGCGTCTACCAGAACGATTGGTAC
AGCCAGTGCGTGCCTGGCGCGGCGTCGACAACGCTGCAGACATCGACCACGTCCAGGCCCA
CCGCCACCAGCACCGCCCCTCCGTCGTCCACCACCTCGCCTAGCGTGGCCAGTCCTATTCG
TCGAGAGGTCTCGCAGGATCTGTTTAACCAGTTCAATCTCTTTGCACAGTATTCTGCAGCC
GCATACTGCGGAAAAAACAATGATGCCCCAGCTGGTACAAACATTACGTGCACGGGAAATG
CCTGCCCCGAGGTAGAGAAGGCGGATGCAACGTTTCTCTACTCGTTTGAAGACTCTGGAGT
GGGCGATGTCACCGGCTTCCTTGCTCTCGACAACACGAACAAATTGATCGTCCTCTCTTTC
CGTGGCTCTCGTTCCATAGAGAACTGGATCGGGAATCTTAAGTTCCTCTTGAAAAAAATAA
ATGACATTTGCTCCGGCTGCAGGGGACATGACGGCTTCACTTCGTCCTGGAGGTCTGTAGC
CGATACGTTAAGGCAGAAGGTGGAGGATGCTGTGAGGGAGCATCCCGACTATCGCGTGGTG
TTTACCGGACATAGCTTGGGTGGTGCATTGGCAACTGTTGCCGGAGCAGACCTGCGTGGAA
ATGGGTATGATATCGACGTGTTTTCATATGGCGCCCCCCGAGTCGGAAACAGGGCTTTTGC
AGAATTCCTGACCGTACAGACCGGCGGAACACTCTACCGCATTACCCACACCAATGATATT
GTCCCTAGACTCCCGCCGCGCGAATTCGGTTACAGCCATTCTAGCCCAGAATACTGGATCA
AATCTGGAACCCTTGTCCCCGTCACCCGAAACGATATCGTGAAGATAGAAGGCATCGATGC
CACCGGCGGCAATAACCGGCCGAACATTCCGGATATCCCTGCGCACCTATGGTACTTCGGG
TTAATTGGGACATGTCTTTAGTGGCCGGCGCGGCTGGGTCGACTCTAGCGAGCTCGAGATC
TAGAGGGTGACTGACACCTGGCGGTAGACAATCAATCCATTTCGCTATAGTTAAAGGATGG
GGATGAGGGCAATTGGTTATATGATCATGTATGTAGTGGGTGTGCATAATAGTAGTGAAAT
GGAAGCCAAGTCATGTGATTGTAATCGACCGACGGAATTGAGGATATCCGGAAATACAGAC
ACCGTGAAAGCCATGGTCTTTCCTTCGTGTAGAAGACCAGACAGACAGTCCCTGATTTACC
CTTGCACAAAGCACTAGAAAATTAGCATTCCATCCTTCTCTGCTTGCTCTGCTGATATCAC
TGTCATTCAATGCATAGCCATGAGCTCATCTTAGATCCAAGCACGTAATTCCATAGCCGAG
GTCCACAGTGGAGCAGCAACATTCCCCATCATTGCTTTCCCCAGGGGCCTCCCAACGACTA

AATCAAGAGTATATCTCTACCGTCCAATAGATCGTCTTCGCTTCAAAATCTTTGACAATTC
CAAGAGGGTCCCCATCCATCAAACCCAGTTCAATAATAGCCGAGATGCATGGTGGAGTCAA
TTAGGCAGTATTGCTGGAATGTCGGGCCAGTTGGCCCGGGTGGTCATTGGCCGCCTGTGAT
GCCATCTGCCACTAAATCCGATCATTGATCCACCGCCCACGAGGCGCGTCTTTGCTTTTTG
CGCGGCGTCCAGGTTCAACTCTCTCGCTCTAGATATCGATGAATTCACTGGCCGTCGTTTT
ACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCC
CCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGC
GCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTAT
TTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAG
CCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCG
CTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATC
ACCGAAACGCGCGAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATG
ATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTA
TTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATA
AATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTT
ATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAG
TAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAG
CGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAA
GTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCC
GCATACACTATTCTCAGAATGACTTGGTTGACGCGTCACCAGTCACAGAAAAGCATCTTAC
GGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCG
GCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACA
TGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAA
CGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACT
GGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAG
TTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGG
AGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCC
CGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGA
TCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATA
TATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTT
TTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACC
CCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTT
GCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACT
CTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGT
AGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCT
AATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCA
AGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGC
CCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAG
CGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACA
GGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGT
TTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATG
GAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCAC
ATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAG
CTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGA
AGAGAG

SEQ ID No. 10:

GCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCAC
GACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCA

CTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGT
GAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGC
CTGCAGGTCGACGCATTCCGAATACGAGGCCTGATTAATGATTACATACGCCTCCGGGTAG
TAGACCGAGCAGCCGAGCCAGTTCAGCGCCTAAAACGCCTTATACAATTAAGCAGTTAAAG
AAGTTAGAATCTACGCTTAAAAAGCTACTTAAAAATCGATCTCGCAGTCCCGATTCGCCTA
TCAAAACCAGTTTAAATCAACTGATTAAAGGTGCCGAACGAGCTATAAATGATATAACAAT
ATTAAAGCATTAATTAGAGCAATATCAGGCCGCGCACGAAAGGCAACTTAAAAAGCGAAAG
CGCTCTACTAAACAGATTACTTTTGAAAAAGGCACATCAGTATTTAAAGCCCGAATCCTTA
TTAAGCGCCGAAATCAGGCAGATAAAGCCATACAGGCAGATAGACCTCTACCTATTAAATC
GGCTTCTAGGCGCGCTCCATCTAAATGTTCTGGCTGTGGTGTACAGGGGCATAAAATTACG
CACTACCCGAATCGATAGAACTACTCATTTTTATATAGAAGTCAGAATTCATAGTGTTTTG
ATCATTTTAAATTTTTATATGGCGGGTGGTGGGCAACTCGCTTGCGCGGGCAACTCGCTTA
CCGATTACGTTAGGGCTGATATTTACGTGAAAATCGTCAAGGGATGCAAGACCAAAGTAGT
AAAACCCCGGAAGTCAACAGCATCCAAGCCCAAGTCCTTCACGGAGAAACCCCAGCGTCCA
CATCACGAGCGAAGGACCACCTCTAGGCATCGGACGCACCATCCAATTAGAAGCAGCAAAG
CGAAACAGCCCAAGAAAAAGGTCGGCCCGTCGGCCTTTTCTGCAACGCTGATCACGGGCAG
CGATCCAACCAACACCCTCCAGAGTGACTAGGGGCGGAAATTTAAAGGGATTAATTTCCAC
TCAACCACAAATCACAGTCGTCCCCGGTATTGTCCTGCAGAATGCAATTTAAACTCTTCTG
CGAATCGCTTGGATTCCCCGCCCCTAGTCGTAGAGCTTAAAGTATGTCCCTTGTCGATGCG
ATGATACACAACATATAAATACTAGCAAGGGATGCCATGCTTGGAGGATAGCAACCGACAA
CATCACATCAAGCTCTCCCTTCTCTGAACAATAAACCCCACAGGGGGGATCCACTAGTAAC
GGCCGCCAGTGTGCTGGAAAGCGACTTGAAACGCCCCAAATGAAGTCCTCCATCCTCGCCA
GCGTCTTCGCCACGGGCGCCGTGGCTCAAAGTGGTCCGTGGCAGCAATGTGGTGGCATCGG
ATGGCAAGGATCGACCGACTGTGTGTCGGGCTACCACTGCGTCTACCAGAACGATTGGTAC
AGCCAGTGCGctagcCCTccTCGTCGAcctGTCTCGCAGGATCTGTTTAACCAGTTCAATC
TCTTTGCACAGTATTCTGCAGCCGCATACTGCGGAAAAAACAATGATGCCCCAGCTGGTAC
AAACATTACGTGCACGGGAAATGCCTGCCCCGAGGTAGAGAAGGCGGATGCAACGTTTCTC
TACTCGTTTGAAGACTCTGGAGTGGGCGATGTCACCGGCTTCCTTGCTCTCGACAACACGA
ACAAATTGATCGTCCTCTCTTTCCGTGGCTCTCGTTCCATAGAGAACTGGATCGGGAATCT
TAAGTTCCTCTTGAAAAAAATAAATGACATTTGCTCCGGCTGCAGGGGACATGACGGCTTC
ACTTCGTCCTGGAGGTCTGTAGCCGATACGTTAAGGCAGAAGGTGGAGGATGCTGTGAGGG
AGCATCCCGACTATCGCGTGGTGTTTACCGGACATAGCTTGGGTGGTGCATTGGCAACTGT
TGCCGGAGCAGACCTGCGTGGAAATGGGTATGATATCGACGTGTTTTCATATGGCGCCCCC
CGAGTCGGAAACAGGGCTTTTGCAGAATTCCTGACCGTACAGACCGGCGGAACACTCTACC
GCATTACCCACACCAATGATATTGTCCCTAGACTCCCGCCGCGCGAATTCGGTTACAGCCA
TTCTAGCCCAGAATACTGGATCAAATCTGGAACCCTTGTCCCCGTCACCCGAAACGATATC
GTGAAGATAGAAGGCATCGATGCCACCGGCGGCAATAACCGGCCGAACATTCCGGATATCC
CTGCGCACCTATGGTACTTCGGGTTAATTGGGACATGTCTTTAGTGGCCGGCGCGGCTGGG
TCGACTCTAGCGAGCTCGAGATCTAGAGGGTGACTGACACCTGGCGGTAGACAATCAATCC
ATTTCGCTATAGTTAAAGGATGGGGATGAGGGCAATTGGTTATATGATCATGTATGTAGTG
GGTGTGCATAATAGTAGTGAAATGGAAGCCAAGTCATGTGATTGTAATCGACCGACGGAAT
TGAGGATATCCGGAAATACAGACACCGTGAAAGCCATGGTCTTTCCTTCGTGTAGAAGACC
AGACAGACAGTCCCTGATTTACCCTTGCACAAAGCACTAGAAAATTAGCATTCCATCCTTC
TCTGCTTGCTCTGCTGATATCACTGTCATTCAATGCATAGCCATGAGCTCATCTTAGATCC
AAGCACGTAATTCCATAGCCGAGGTCCACAGTGGAGCAGCAACATTCCCCATCATTGCTTT
CCCCAGGGGCCTCCCAACGACTAAATCAAGAGTATATCTCTACCGTCCAATAGATCGTCTT
CGCTTCAAAATCTTTGACAATTCCAAGAGGGTCCCCATCCATCAAACCCAGTTCAATAATA
GCCGAGATGCATGGTGGAGTCAATTAGGCAGTATTGCTGGAATGTCGGGCCAGTTGGCCCG
GGTGGTCATTGGCCGCCTGTGATGCCATCTGCCACTAAATCCGATCATTGATCCACCGCCC
ACGAGGCGCGTCTTTGCTTTTTGCGCGGCGTCCAGGTTCAACTCTCTCGCTCTAGATATCG
ATGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAAC
TTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCAC

CGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTT
CTCCTTACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCT
CTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACG
GGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATG
TGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGACGAAAGGGCCTCGTGATACGCC
TATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCG
GGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCG
CTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTA
TTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGC
TCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGT
TACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTT
TTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGC
CGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGACGCGTCA
CCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCA
TAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGA
GCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCG
GAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAA
CAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAAT
AGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGC
TGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCAC
TGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAAC
TATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAA
CTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTA
AAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTT
TTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTT
TTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTT
TGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGAT
ACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCA
CCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGT
CGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTG
AACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATAC
CTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATC
CGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTG
GTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGC
TCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGG
CCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAA
CCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGC
GAGTCAGTGAGCGAGGAAGCGGAAGAGAG

SEQ ID No. 11:

GCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCAC
GACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCA
CTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGT
GAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGC
CTGCAGGTCGACGCATTCCGAATACGAGGCCTGATTAATGATTACATACGCCTCCGGGTAG
TAGACCGAGCAGCCGAGCCAGTTCAGCGCCTAAAACGCCTTATACAATTAAGCAGTTAAAG
AAGTTAGAATCTACGCTTAAAAAGCTACTTAAAAATCGATCTCGCAGTCCCGATTCGCCTA
TCAAAACCAGTTTAAATCAACTGATTAAAGGTGCCGAACGAGCTATAAATGATATAACAAT
ATTAAAGCATTAATTAGAGCAATATCAGGCCGCGCACGAAAGGCAACTTAAAAAGCGAAAG
CGCTCTACTAAACAGATTACTTTTGAAAAAGGCACATCAGTATTTAAAGCCCGAATCCTTA

TTAAGCGCCGAAATCAGGCAGATAAAGCCATACAGGCAGATAGACCTCTACCTATTAAATC
GGCTTCTAGGCGCGCTCCATCTAAATGTTCTGGCTGTGGTGTACAGGGGCATAAAATTACG
CACTACCCGAATCGATAGAACTACTCATTTTTATATAGAAGTCAGAATTCATAGTGTTTTG
ATCATTTTAAATTTTTATATGGCGGGTGGTGGGCAACTCGCTTGCGCGGGCAACTCGCTTA
CCGATTACGTTAGGGCTGATATTTACGTGAAAATCGTCAAGGGATGCAAGACCAAAGTAGT
AAAACCCCGGAAGTCAACAGCATCCAAGCCCAAGTCCTTCACGGAGAAACCCCAGCGTCCA
CATCACGAGCGAAGGACCACCTCTAGGCATCGGACGCACCATCCAATTAGAAGCAGCAAAG
CGAAACAGCCCAAGAAAAAGGTCGGCCCGTCGGCCTTTTCTGCAACGCTGATCACGGGCAG
CGATCCAACCAACACCCTCCAGAGTGACTAGGGGCGGAAATTTAAAGGGATTAATTTCCAC
TCAACCACAAATCACAGTCGTCCCCGGTATTGTCCTGCAGAATGCAATTTAAACTCTTCTG
CGAATCGCTTGGATTCCCCGCCCCTAGTCGTAGAGCTTAAAGTATGTCCCTTGTCGATGCG
ATGATACACAACATATAAATACTAGCAAGGGATGCCATGCTTGGAGGATAGCAACCGACAA
CATCACATCAAGCTCTCCCTTCTCTGAACAATAAACCCCACAGGGGGGATCCACTAGTAAC
GGCCGCCAGTGTGCTGGAAAGCGACTTGAAACGCCCCAAATGAAGTCCTCCATCCTCGCCA
GCGTCTTCGCCACGGGCGCCGTGGCTCAAAGTGGTCCGTGGCAGCAATGTGGTGGCATCGG
ATGGCAAGGATCGACCGACTGTGTGTCGGGCTACCACTGCGTCTACCAGAACGATTGGTAC
AGCCAGTGCGCTAGCGTCCAGATCCCCTCCAGCAGCACCAGCTCTCCGGTCAACCAGCCTA
CCAGCACCAGCACCACGTCCACCTCCACCACCTCGAGCCCGCCAGTCCAGCCTACGACTCC
CAGCGCTAGCCCTCCTCGTCGACCTGTCTCGCAGGATCTGTTTAACCAGTTCAATCTCTTT
GCACAGTATTCTGCAGCCGCATACTGCGGAAAAAACAATGATGCCCCAGCTGGTACAAACA
TTACGTGCACGGGAAATGCCTGCCCCGAGGTAGAGAAGGCGGATGCAACGTTTCTCTACTC
GTTTGAAGACTCTGGAGTGGGCGATGTCACCGGCTTCCTTGCTCTCGACAACACGAACAAA
TTGATCGTCCTCTCTTTCCGTGGCTCTCGTTCCATAGAGAACTGGATCGGGAATCTTAAGT
TCCTCTTGAAAAAAATAAATGACATTTGCTCCGGCTGCAGGGGACATGACGGCTTCACTTC
GTCCTGGAGGTCTGTAGCCGATACGTTAAGGCAGAAGGTGGAGGATGCTGTGAGGGAGCAT
CCCGACTATCGCGTGGTGTTTACCGGACATAGCTTGGGTGGTGCATTGGCAACTGTTGCCG
GAGCAGACCTGCGTGGAAATGGGTATGATATCGACGTGTTTTCATATGGCGCCCCCCGAGT
CGGAAACAGGGCTTTTGCAGAATTCCTGACCGTACAGACCGGCGGAACACTCTACCGCATT
ACCCACACCAATGATATTGTCCCTAGACTCCCGCCGCGCGAATTCGGTTACAGCCATTCTA
GCCCAGAATACTGGATCAAATCTGGAACCCTTGTCCCCGTCACCCGAAACGATATCGTGAA
GATAGAAGGCATCGATGCCACCGGCGGCAATAACCGGCCGAACATTCCGGATATCCCTGCG
CACCTATGGTACTTCGGGTTAATTGGGACATGTCTTTAGTGGCCGGCGCGGCTGGGTCGAC
TCTAGCGAGCTCGAGATCTAGAGGGTGACTGACACCTGGCGGTAGACAATCAATCCATTTC
GCTATAGTTAAAGGATGGGGATGAGGGCAATTGGTTATATGATCATGTATGTAGTGGGTGT
GCATAATAGTAGTGAAATGGAAGCCAAGTCATGTGATTGTAATCGACCGACGGAATTGAGG
ATATCCGGAAATACAGACACCGTGAAAGCCATGGTCTTTCCTTCGTGTAGAAGACCAGACA
GACAGTCCCTGATTTACCCTTGCACAAAGCACTAGAAAATTAGCATTCCATCCTTCTCTGC
TTGCTCTGCTGATATCACTGTCATTCAATGCATAGCCATGAGCTCATCTTAGATCCAAGCA
CGTAATTCCATAGCCGAGGTCCACAGTGGAGCAGCAACATTCCCCATCATTGCTTTCCCCA
GGGGCCTCCCAACGACTAAATCAAGAGTATATCTCTACCGTCCAATAGATCGTCTTCGCTT
CAAAATCTTTGACAATTCCAAGAGGGTCCCCATCCATCAAACCCAGTTCAATAATAGCCGA
GATGCATGGTGGAGTCAATTAGGCAGTATTGCTGGAATGTCGGGCCAGTTGGCCCGGGTGG
TCATTGGCCGCCTGTGATGCCATCTGCCACTAAATCCGATCATTGATCCACCGCCCACGAG
GCGCGTCTTTGCTTTTTGCGCGGCGTCCAGGTTCAACTCTCTCGCTCTAGATATCGATGAA
TTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAAT
CGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATC
GCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCT
TACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGAT
GCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTT
GTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCA
GAGGTTTTCACCGTCATCACCGAAACGCGCGAGACGAAAGGGCCTCGTGATACGCCTATTT
TTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAA

ATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCAT
GAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAA
CATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACC
CAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACAT
CGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCA
ATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGC
AAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGACGCGTCACCAGT
CACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACC
ATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAA
CCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCT
GAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACG
TTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACT
GGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTT
TATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGG
CCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGG
ATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTC
AGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGG
ATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGT
TCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCT
GCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCG
GATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAA
ATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCC
TACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGT
CTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGG
GGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACA
GCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTA
AGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATC
TTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTC
AGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTT
TGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTA
TTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTC
AGTGAGCGAGGAAGCGGAAGAGAG

SEQ ID No. 12:

GAGAAAAAACTATAGGATCCACTAGTAACGGCCGCCAGTGTGCTCTAAAGACTATGAAGC
TCTCGCTTTTGTCCACCTTCGCTGCTGTCATCATCGGTGCCCTCGCTCTACCCCAGGGTT
GCACTGCTGAGAGGTGGGCTCAGTGCGGCGGCAATGGCTGGAGCGGCTGCACCACCTGCG
TCGCTGGCAGCACTTGCACGAAGATTAATGACTGGTACCATCAGTGCCTGCCCTCCAGCA
GCACCAGCTCTCCGGTCAACCAGCCTACCAGCACCAGCACCACGTCCACCTCCACCACCT
CGAGCCCGCCAGTCCAGCCTACGACTCCCAGCGGCCAGGGTCCTGGAGGAGGCGGGTCAG
TCACTTGCCCCGGTGGACAGTCCACTTCGAACAGCCAGTGCTGCGTCTGGTTCGACGTTC
TAGACGATCTTCAGACCAACTTCTACCAAGGGTCCAAGTGTGAGAGCCCTGTTCGCAAGA
TTCTTAGAATTGTTTTCCATGACGCGATCGGATTTTCGCCGGCGTTGACTGCTGCTGGTC

AATTCGGTGGTGGAGGAGCTGATGGCTCCATCATTGCGCATTCGAACATCGAATTGGCCT
TCCCGGCTAATGGCGGCCTCACCGACACCGTCGAAGCCCTCCGCGCGGTCGGTATCAACC
ACGGTGTCTCTTTCGGCGATCTCATCCAATTCGCCACTGCCGTCGGCATGTCCAACTGCC

CTGGCTCTCCCCGACTTGAGTTCTTGACGGGCAGGAGCAACAGTTCCCAACCCTCCCCTC
CTTCGTTGATCCCCGGTCCCGGAAACACGGTCACCGCTATCTTGGATCGTATGGGCGATG
CAGGCTTCAGCCCTGATGAAGTAGTCGACTTGCTTGCTGCGCATAGTTTGGCTTCTCAGG
AGGGTTTGAACTCGGCCATCTTCAGATCTCCTTTGGACTCGACCCCTCAAGTTTTCGATA
CCCAGTTCTACATTGAGACCTTGCTCAAGGGTACCACTCAGCCTGGCCCTTCTCTCGGCT
TTGCAGAGGAGCTCTCCCCCTTCCCTGGCGAATTCCGCATGAGGTCCGATGCTCTCTTGG
CTCGCGACTCCCGAACCGCCTGCCGATGGCAATCCATGACCAGCAGCAATGAAGTTATGG
GCCAGCGATACCGCGCCGCCATGGCCAAGATGTCTGTTCTCGGCTTCGACAGGAACGCCC
TCACCGATTGCTCTGACGTTATTCCTTCTGCTGTGTCCAACAACGCTGCTCCTGTTATCC
CTGGTGGCCTTACTGTCGATGATATCGAGGTTTCGTGCCCGAGCGAGCCTTTCCCTGAAA
TTGCTACCGCCTCAGGCCCTCTCCCCTCCCTCGCTCCTGCTCCTTGATCTGGTGAAGATG
GTACATCCTGCTCTCTCATCATCCCTCTTAGCTATTTATCCAATCTATCTACCTATCTAT
GCAGTTTCTGTTCTATCACCACAGGAAGCAAGAAAGAAAAACAACAATGCAACGTGAGCA
GAAATCAGCAAAAAAATAAATCAGTATACTACAGTAATGAGGCCAGTTTGCGTGGTGTCA
GAAGTAAGTACGACTCGGCTTTACACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCC

SEQ ID No. 13:

GAGAAAAAACTATAGGATCCACTAGTAACGGCCGCCAGTGTGCTCTAAAGACTATGAAGC
TCTCGCTTTTGTCCACCTTCGCTGCTGTCATCATCGGTGCCCTCGCTCTACCCCAAGGTT
GCACTGCTGAGAGGTGGGCTCAGTGCGGCGGCAATGGCTGGAGCGGCTGCACCACCTGCG
TCGCTGGCAGCACTTGCACGAAGATTAATGACTGGTATCATCAGTGCCTGCCCTCCAGCA
GCACCAGCTCTCCGGTCAACCAGCCTACCAGCACCAGCACCACGTCCACCTCCACCACCT
CGAGCCCGCCAGTCCAGCCTACGACTCCGAGCGGTCAGGGTCCTGGAGGAGGCGGGTCAG
TCACTTGCCCCGGTGGACAGTCCACTTCGAACAGCCAGTGCTGCGTCTGGTTCGACGTTC
TAGACGATCTTCAGACCAACTTCTACCAAGGGTCCAAGTGTGAGAGCCCTGTTCGCAAGA
TTCTTAGAATTGTTTTCCATGACGCGATCGGATTTTCGCCGGCGTTGACTGCTGCTGGTC
AATTCGGTGGTGGAGGAGCTGATGGCTCCATCATTGCGCATTCGAACATCGAATTGGCCT
TCCCGGCTAATGGCGGCCTCACCGACACCGTCGAAGCCCTCCGCGCGGTCGGTATCAACC
ACGGTGTCTCTTTCGGCGATCTCATCCAATTCGCCACTGCCGTCGGCATGTCCAACTGCC

CTGGCTCTCCCCGACTTGAGTTCTTGACGGGCAGGAGCAACAGTTCCCAACCCTCCCCTC
CTTCGTTGATCCCCGGTCCCGGAAACACGGTCACCGCTATCTTGGATCGTATGGGCGATG
CAGGCTTCAGCCCTGATGAAGTAGTCGACTTGCTTGCTGCGCATAGTTTGGCTTCTCAGG
AGGGTTTGAACTCGGCCATCTTCAGATCTCCTTTGGACTCGACCCCTCAAGTTTTCGATA
CCCAGTTCTACATTGAGACCTTGCTCAAGGGTACCACTCAGCCTGGCCCTTCTCTCGGCT
TTGCAGAGGAGCTCTCCCCCTTCCCTGGCGAATTCCGCATGAGGTCCGATGCTCTCTTGG
CTCGCGACTCCCGAACCGCCTGCCGATGGCAATCCATGACCAGCAGCAATGAAGTTATGG
GCCAGCGATACCGCGCCGCCATGGCCAAGATGTCTGTTCTCGGCTTCGACAGGAACGCCC
TCACCGATTGCTCTGACGTTATTCCTTCTGCTGTGTCCAACAACGCTGCTCCTGTTATCC
CTGGTGGCCTTACTGTCGATGATATCGAGGTTTCGTGCCCGAGCGAGCCTTTCCCTGAAA
TTGCTACCGCCTCAGGCCCTCTCCCCTCCCTCGCTCCTGCTCCTTGATCTGGTGAAGATG
GTACATCCTGCTCTCTCATCATCCCTCTTAGCTATTTATCCAATCTATCTACCTATCTAT
GCAGTTTCTGTTCTATCACCACAGGAAGCAAGAAAGAAAAACAACAATGCAACGTGAGCA
GAAATCAGCAAAAAAATAAATCAGTATACTACAGTAATGAGGCCAGTTTGCGTGGTGTCA
GAAGTAAGTACGACTCGGCTTTACACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCC

SEQ ID No. 14:

GAGAAAAAACTATAGGATCCACTAGTAACGGCCGCCAGTGTGCTCTAAAGACTATGAAGC
TCTCGCTTTTGTCCACCTTCGCTGCTGTCATCATCGGTGCCCTCGCTCTACCCCAAGGTT
GCACTGCTGAGAGGTGGGCTCAGTGCGGCGGCAATGGCTGGAGCGGCTGCACCACCTGCG
TCGCTGGCAGCACTTGCACGAAGATTAATGACTGGTACCATCAGTGCCTGCCCTCCTCCA
GCACCAGCTCTCCGGTCAACCAGCCTACCAGCACCAGCTCCAGCCCTCCAGTCCAGCCTA
CGACTCCTAGCGGACAAGGTCCTGGAGGAGGCGGGTCAGTCACTTGCCCCGGTGGACAGT
CCACTTCGAACAGCCAGTGCTGCGTCTGGTTCGACGTTCTAGACGATCTTCAGACCAACT
TCTACCAAGGGTCCAAGTGTGAGAGCCCTGTTCGCAAGATTCTTAGAATTGTTTTCCATG

SEQ ID No. 15:

GAGAAAAAACTATAGGATCCACTAGTAACGGCCGCCAGTGTGCTCTAAAGACTATGAAGC
TCTCGCTTTTGTCCACCTTCGCTGCTGTCATCATCGGTGCCCTCGCTCTACCCCAAGGTT
GCACTGCTGAGAGGTGGGCTCAGTGCGGCGGCAATGGCTGGAGCGGCTGCACCACCTGCG

TCGCTGGCAGCACTTGCACGAAGATTAATGACTGGTACCATCAGTGCCTCGCCCCCGTCG
TCGCCCCCGCCCCCGCCCCCGCCCCCCAAGGTCCTGGAGGAGGCGGGTCAGTCACTTGCC
CCGGTGGACAGTCCACTTCGAACAGCCAGTGCTGCGTCTGGTTCGACGTTCTAGACGATC
TTCAGACCAACTTCTACCAAGGGTCCAAGTGTGAGAGCCCTGTTCGCAAGATTCTTAGAA
TTGTTTTCCATGACGCGATCGGATTTTCGCCGGCGTTGACTGCTGCTGGTCAATTCGGTG

SEQ ID No. 16:

GAGAAAAAACTATAGGATCCACTAGTAACGGCCGCCAGTGTGCTCTAAAGACTATGAAGC
TCTCGCTTTTGTCCACCTTCGCTGCTGTCATCATCGGTGCCCTCGCTCTACCCCAAGGTT
GCACTGCTGAGAGGTGGGCTCAGTGCGGCGGCAATGGCTGGAGCGGCTGCACCACCTGCG
TCGCTGGCAGCACTTGCACGAAGATTAATGACTGGTACCATCAGTGCCTGCAAGCCCCCC
AACAGAGCCCCCGCATCGAACGTCCACGCGCTCAGGGTCCTGGAGGAGGCGGGTCAGTCA
CTTGCCCCGGTGGACAGTCCACTTCGAACAGCCAGTGCTGCGTCTGGTTCGACGTTCTAG
ACGATCTTCAGACCAACTTCTACCAAGGGTCCAAGTGTGAGAGCCCTGTTCGCAAGATTC
TTAGAATTGTTTTCCATGACGCGATCGGATTTTCGCCGGCGTTGACTGCTGCTGGTCAAT

SEQ ID No. 17:

```
CTGGGGTAATTAATCAGCGAAGCGATGATTTTTGATCTATTAACAGATATATAAATGCAA 60
AAACTGCATAACCACTTTAACTAATACTTTCAACATTTTCGGTTTGTATTACTTCTTATT 120
CAAATGTAATAAAAGTATCAACAAAAAATTGTTAATATACCTCTATACTTTAACGTCAAG 180
GAGAAAAAACTATAGGATCCACTAGTAACGGCCGCCAGTGTGCTCTAAAGACTATGAAGC 240
TCTCGCTTTTGTCCACCTTCGCTGCTGTCATCATCGGTGCCCTCGCTCTACCCCAGGGTT 300
GCACTGCTGAGAGGTGGGCTCAGTGCGGCGGCAATGGCTGGAGCGGCTGCACCACCTGCG 360
TCGCTGGCAGCACTTGCACGAAGATTAATGACTGGTACCATCAGTGCCTGCAAGCCCCCC 420
AACAGAGCCCCCGCATCGAACGTCCACGCGCTCAGCAGAGCTGCAACACCCCCAGCAACC 480
GGGCGTGCTGGACTGACGGATACGACATCAACACCGACTACGAAGTGGACAGCCCGGACA 540
CGGGTGTTGTTCGGCCTTATACTCTGACTCTCACCGAAGTCGACAACTGGACCGGACCTG 600
ATGGCGTCGTCAAGGAGAAGGTCATGCTGGTTAACAATAGTATAATCGGACCAACAATCT 660
TTGCGGACTGGGGCGACACGATCCAGGTAACGGTCATCAACAACCTCGAGACCAACGGCA 720
CGTCGATCCACTGGCACGGACTGCACCAGAAGGGCACCAACCTGCACGACGGCGCCAACG 780
GTATCACCGAGTGCCCGATCCCGCCCAAGGGAGGGAGGAAGGTGTACCGGTTCAAGGCTC 840
AGCAGTACGGGACGAGCTGGTACCACTCGCACTTCTCGGCCCAGTACGGCAACGGCGTGG 900
TCGGGGCCATTCAGATCAACGGGCCGGCCTCGCTGCCGTACGACACCGACCTGGGCGTGT 960
TCCCCATCAGCGACTACTACTACAGCTCGGCCGACGAGCTGGTGGAACTCACCAAGAACT 1020
CGGGCGCGCCCTTCAGCGACAACGTCCTGTTCAACGGCACGGCCAAGCACCCGGAGACGG 1080
GCGAGGGCGAGTACGCCAACGTGACGCTCACCCCGGGCCGGCGGCACCGCCTGCGCCTGA 1140
TCAACACGTCGGTCGAGAACCACTTCCAGGTCTCGCTCGTCAACCACACCATGACCATCA 1200
TCGCCGCCGACATGGTGCCCGTCAACGCCATGACGGTCGACAGCCTCTTCCTCGGCGTCG 1260
GCCAGCGCTACGATGTCGTCATCGAAGCCAGCCGAACGCCCGGGAACTACTGGTTTAACG 1320
TCACATTTGGCGGCGGCCTGCTCTGCGGCGGCTCCAGGAATCCCTACCCGGCCGCCATCT 1380
TCCACTACGCCGGCGCCCCCGGCGGCCCGCCCACGGACGAGGGCAAGGCCCCGGTCGACC 1440
ACAACTGCCTGGACCTCCCCAACCTCAAGCCCGTCGTGGCCCGCGACGTGCCCCTGAGCG 1500
GCTTCGCCAAGCGGCCCGACAACACGCTCGACGTCACCCTCGACACCACGGGCACGCCCC 1560
TGTTCGTCTGGAAGGTCAACGGCAGCGCCATCAACATCGACTGGGGCAGGCCCGTCGTCG 1620
ACTACGTCCTCACGCAGAACACCAGCTTCCCACCCGGGTACAACATTGTCGAGGTGAACG 1680
```

GAGCTGATCAGTGGTCGTACTGGTTGATCGAGAATGATCCCGGCGCACCTTTCACCCTAC 1740
CGCATCCGATGCACCTGCACGGCCACGACTTTTACGTGCTGGGCCGCTCGCCCGACGAGT 1800
CGCCGGCATCCAACGAGCGGCACGTGTTCGATCCGGCGCGGGACGCGGGCCTGCTGAGCG 1860
GGGCCAACCCTGTGCGGCGGGACGTGACGATGCTGCCGGCGTTCGGGTGGGTGGTGCTGG 1920
CCTTCCGGGCCGACAACCCGGGCGCCTGGCTGTTCCACTGCCACATCGCCTGGCACGTCT 1980
CGGGCGGCCTGGGCGTCGTCTACCTCGAGCGCGCCGACGACCTGCGCGGGGCCGTCTCGG 2040
ACGCCGACGCCGACGACCTCGACCGCCTCTGCGCCGACTGGCGCCGCTACTGGCCTACCA 2100
ACCCCTACCCCAAGTCCGACTCGGGCCTCAAGCACCGCTGGGTCGAGGAGGGCGAGTGGC 2160
TGGTCAAGGCGTGAGCGAAGGAGGAAAAAGGCGGCCGCATAGTATAGGCCGCTCGAGCAT 2220
GCATCTAGAGGGCCGCATCATGTAATTAGTTATGTCACGCTTACATTCACGCCCTCCCC 2279

SEQ ID No.18:

CTGGGGTAATTAATCAGCGAAGCGATGATTTTTGATCTATTAACAGATATATAAATGCAA 60
AAACTGCATAACCACTTTAACTAATACTTTCAACATTTTCGGTTTGTATTACTTCTTATT 120
CAAATGTAATAAAAGTATCAACAAAAAATTGTTAATATACCTCTATACTTTAACGTCAAG 180
GAGAAAAAACTATAGGATCCCCAACATGAGGTCCTTCATCAGCGCCGCGACGCTTTTGGT 240
GGGCATTCTCACCCCTAGCGTTGCTGCTGCCCCTCCATCCACCCCTGAGCAGCGCGACCT 300
GCTCGTCCCGATCACGGAGAGGGAGGAGGCAGCCGTGAAGGCTCGCCAGCAGAGCTGCAA 360
CACCCCCAGCAACCGGGCGTGCTGGACTGACGGATACGACATCAACACCGACTACGAAGT 420
GGACAGCCCGGACACGGGTGTTGTTCGGCCTTATACTCTGACTCTCACCGAAGTCGACAA 480
CTGGACCGGACCTGATGGCGTCGTCAAGGAGAAGGTCATGCTGGTTAACAATAGTATAAT 540
CGGACCAACAATCTTTGCGGACTGGGGCGACACGATCCAGGTAACGGTCATCAACAACCT 600
CGAGACCAACGGCACGTCGATCCACTGGCACGGACTGCACCAGAAGGGCACCAACCTGCA 660
CGACGGCGCCAACGGTATCACCGAGTGCCCGATCCCGCCCAAGGGAGGGAGGAAGGTGTA 720
CCGGTTCAAGGCTCAGCAGTACGGGACGAGCTGGTACCACTCGCACTTCTCGGCCCAGTA 780
CGGCAACGGCGTGGTCGGGGCCATTCAGATCAACGGGCCGGCCTCGCTGCCGTACGACAC 840
CGACCTGGGCGTGTTCCCCATCAGCGACTACTACTACAGCTCGGCCGACGAGCTGGTGGA 900
ACTCACCAAGAACTCGGGCGCGCCCTTCAGCGACAACGTCCTGTTCAACGGCACGGCCAA 960
GCACCCGGAGACGGGCGAGGGCGAGTACGCCAACGTGACGCTCACCCCGGGCCGGCGGCA 1020
CCGCCTGCGCCTGATCAACACGTCGGTCGAGAACCACTTCCAGGTCTCGCTCGTCAACCA 1080
CACCATGACCATCATCGCCGCCGACATGGTGCCCGTCAACGCCATGACGGTCGACAGCCT 1140
CTTCCTCGGCGTCGGCCAGCGCTACGATGTCGTCATCGAAGCCAGCCGAACGCCCGGGAA 1200
CTACTGGTTTAACGTCACATTTGGCGGCGGCCTGCTCTGCGGCGGCTCCAGGAATCCCTA 1260
CCCGGCCGCCATCTTCCACTACGCCGGCGCCCCCGGCGGCCCGCCCACGGACGAGGGCAA 1320
GGCCCCGGTCGACCACAACTGCCTGGACCTCCCCAACCTCAAGCCCGTCGTGGCCCGCGA 1380
CGTGCCCCTGAGCGGCTTCGCCAAGCGGCCCGACAACACGCTCGACGTCACCCTCGACAC 1440
CACGGGCACGCCCCTGTTCGTCTGGAAGGTCAACGGCAGCGCCATCAACATCGACTGGGG 1500
CAGGCCCGTCGTCGACTACGTCCTCACGCAGAACACCAGCTTCCCACCCGGGTACAACAT 1560
TGTCGAGGTGAACGGAGCTGATCAGTGGTCGTACTGGTTGATCGAGAATGATCCCGGCGC 1620
ACCTTTCACCCTACCGCATCCGATGCACCTGCACGGCCACGACTTTTACGTGCTGGGCCG 1680
CTCGCCCGACGAGTCGCCGGCATCCAACGAGCGGCACGTGTTCGATCCGGCGCGGGACGC 1740
GGGCCTGCTGAGCGGGGCCAACCCTGTGCGGCGGGACGTGACGATGCTGCCGGCGTTCGG 1800
GTGGGTGGTGCTGGCCTTCCGGGCCGACAACCCGGGCGCCTGGCTGTTCCACTGCCACAT 1860
CGCCTGGCACGTCTCGGGCGGCCTGGGCGTCGTCTACCTCGAGCGCGCCGACGACCTGCG 1920
CGGGGCCGTCTCGGACGCCGACGCCGACGACCTCGACCGCCTCTGCGCCGACTGGCGCCG 1980
CTACTGGCCTACCAACCCCTACCCCAAGTCCGAC**TCGGGCCTCAAGCACCGCTGGGTCGA 2040**
GGAGGGCGAGTGGCTGGTCAAGGCGCCCTCCAGCAGCACCAGCTCTCCGGTCAACCAGCC 2100
TACCAGCACCAGCACCACGTCCACCTCCACCACCTCGAGCCCGCCAGTCCAGCCTACGAC 2160
TCCCAGCGGCTGCACTGCTGAGAGGTGGGCTCAGTGCGGCGGCAATGGCTGGAGCGGCTG 2220
CACCACCTGCGTCGCTGGCAGCACTTGCACGAAGATTAATGACTGGTACCATCAGTGCCT 2280
GTAGGCGGCCGCATTCTTAT 2300

SEQ ID No. 19:

CTGGGGTAATTAATCAGCGAAGCGATGATTTTTGATCTATTAACAGATATATAAATGCAA 60
AAACTGCATAACCACTTTAACTAATACTTTCAACATTTTCGGTTTGTATTACTTCTTATT 120
CAAATGTAATAAAAGTATCAACAAAAAATTGTTAATATACCTCTATACTTTAACGTCAAG 180
GAGAAAAAACTATAGGATCCCCAACATGAGGTCCTTCATCAGCGCCGCGACGCTTTTGGT 240

GGGCATTCTCACCCCTAGCGTTGCTGCTGCCCCTCCATCCACCCCTGAGCAGCGCGACCT 300
GCTCGTCCCGATCACGGAGAGGGAGGAGGCAGCCGTGAAGGCTCGCCAGCAGAGCTGCAA 360
CACCCCCAGCAACCGGGCGTGCTGGACTGACGGATACGACATCAACACCGACTACGAAGT 420
GGACAGCCCGGACACGGGTGTTGTTCGGCCTTATACTCTGACTCTCACCGAAGTCGACAA 480
CTGGACCGGACCTGATGGCGTCGTCAAGGAGAAGGTCATGCTGGTTAACAATAGTATAAT 540
CGGACCAACAATCTTTGCGGACTGGGGCGACACGATCCAGGTAACGGTCATCAACAACCT 600
CGAGACCAACGGCACGTCGATCCACTGGCACGGACTGCACCAGAAGGGCACCAACCTGCA 660
CGACGGCGCCAACGGTATCACCGAGTGCCCGATCCCGCCCAAGGGAGGGAGGAAGGTGTA 720
CCGGTTCAAGGCTCAGCAGTACGGGACGAGCTGGTACCACTCGCACTTCTCGGCCCAGTA 780
CGGCAACGGCGTGGTCGGGGCCATTCAGATCAACGGGCCGGCCTCGCTGCCGTACGACAC 840
CGACCTGGGCGTGTTCCCCATCAGCGACTACTACTACAGCTCGGCCGACGAGCTGGTGGA 900
ACTCACCAAGAACTCGGGCGCGCCCTTCAGCGACAACGTCCTGTTCAACGGCACGGCCAA 960
GCACCCGGAGACGGGCGAGGGCGAGTACGCCAACGTGACGCTCACCCCGGGCCGGCGGCA 1020
CCGCCTGCGCCTGATCAACACGTCGGTCGAGAACCACTTCCAGGTCTCGCTCGTCAACCA 1080
CACCATGACCATCATCGCCGCCGACATGGTGCCCGTCAACGCCATGACGGTCGACAGCCT 1140
CTTCCTCGGCGTCGGCCAGCGCTACGATGTCGTCATCGAAGCCAGCCGAACGCCCGGGAA 1200
CTACTGGTTTAACGTCACATTTGGCGGCGGCCTGCTCTGCGGCGGCTCCAGGAATCCCTA 1260
CCCGGCCGCCATCTTCCACTACGCCGGCGCCCCCGGCGGCCCGCCCACGGACGAGGGCAA 1320
GGCCCCGGTCGACCACAACTGCCTGGACCTCCCCAACCTCAAGCCCGTCGTGGCCCGCGA 1380
CGTGCCCCTGAGCGGCTTCGCCAAGCGGCCCGACAACACGCTCGACGTCACCCTCGACAC 1440
CACGGGCACGCCCCTGTTCGTCTGGAAGGTCAACGGCAGCGCCATCAACATCGACTGGGG 1500
CAGGCCCGTCGTCGACTACGTCCTCACGCAGAACACCAGCTTCCCACCCGGGTACAACAT 1560
TGTCGAGGTGAACGGAGCTGATCAGTGGTCGTACTGGTTGATCGAGAATGATCCCGGCGC 1620
ACCTTTCACCCTACCGCATCCGATGCACCTGCACGGCCACGACTTTTACGTGCTGGGCCG 1680
CTCGCCCGACGAGTCGCCGGCATCCAACGAGCGGCACGTGTTCGATCCGGCGCGGGACGC 1740
GGGCCTGCTGAGCGGGGCCAACCCTGTGCGGCGGGACGTGACGATGCTGCCGGCGTTCGG 1800
GTGGGTGGTGCTGGCCTTCCGGGCCGACAACCCGGGCGCCTGGCTGTTCCACTGCCACAT 1860
CGCCTGGCACGTCTCGGGCGGCCTGGGCGTCGTCTACCTCGAGCGCGCCGACGACCTGCG 1920
CGGGGCCGTCTCGGACGCCGACGCCGACGACCTCGACCGCCTCTGCGCCGACTGGCGCCG 1980
CTACTGGCCTACCAACCCCTACCCCAAGTCCGAC
CCCTCCAGCAGCACCAGCTCTCCGGTCAACCAGCC
TACCAGCACCAGCACCACGTCCACCTCCACCACCTCGAGCCCGCCAGTCCAGCCTACGAC
TCCCAGCGGCTGCACTGCTGAGAGGTGGGCTCAGTGCGGCGGCAATGGCTGGAGCGGCTG
CACCACCTGCGTCGCTGGCAGCACTTGCACGAAGATTAATGACTGGTACCATCAGTGCCT
GTAGGCGGCCGCATTCTTAT

CLAIMS

1. A process for removal or bleaching of soiling or stains present on cellulosic fabric, wherein the fabric is contacted in aqueous medium with a modified enzyme (enzyme hybrid) which comprises a catalytically active amino acid sequence of a non-cellulolytic enzyme linked to an amino acid sequence comprising a cellulose-binding domain.

2. A process according to claim 1, wherein said soiling or stain originates from starch, protein, fat, soil, clay, fruit, vegetables, coffee, tea, spices, red wine, body fluids, grass or ink.

3. A process according to claim 1, wherein said catalytically active amino acid sequence derives from an enzyme selected from the group consisting of amylases, proteases, lipases, pectinases and oxidoreductases.

4. A process according to claim 3, wherein said amylase is an α-amylase obtainable from a species of *Bacillus*.

5. A process according to claim 3 or 4, wherein said α-amylase is obtainable from *Bacillus licheniformis*.

6. A process according to claim 3, wherein said protease is obtainable from a species of *Bacillus* or *Fusarium*.

7. A process according to claim 3, wherein said lipase is obtainable from a species of *Humicola*, *Candida*, *Pseudomonas* or *Bacillus*.

8. A process according to claim 3, wherein said oxidoreductase is a peroxidase or a laccase.

9. A process according to claim 8, wherein said peroxidase is obtainable from a species of *Coprinus*.

10. A process according to claim 8 or 9, wherein said peroxidase is obtainable from *C. cinereus*.

11. A process according to claim 8, wherein said laccase is obtainable from a species of *Trametes*, *Myceliophthora*, *Schytalidium* or *Polyporus*.

12. A process according to claim 1, wherein said cellulose-binding domain is obtainable from a cellulase, a xylanase, a mannanase, an arabinofuranosidase, an acetylesterase or a chitinase.

13. A process according to claim 1, wherein said enzyme hybrid is obtained by a method comprising growing a transformed host cell containing an expression cassette which comprises a DNA sequence encoding said enzyme hybrid, whereby said enzyme hybrid is expressed.

14. A detergent composition comprising:

an enzyme hybrid which comprises a catalytically active amino acid sequence of a non-cellulolytic enzyme linked to an amino acid sequence comprising a cellulose-binding domain, and

a surfactant.

15. A process for washing soiled or stained cellulosic fabric, wherein said fabric is washed in an aqueous medium to which is added a detergent composition according to claim 14.

16. An enzyme hybrid encoded by a hybrid-encoding DNA sequence comprised within the DNA sequences of SEQ ID No. 1, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 7, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18 or SEQ ID No. 19.

17. An enzyme hybrid having an amino acid sequence comprised within the amino acid sequences of SEQ ID No. 2, SEQ ID No. 4, SEQ ID No. 6 or SEQ ID No. 8.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00042

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C11D 3/386, C07K 19/00, C12N 9/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, BIOSIS, CA, DBA, MEDLINE, PASCAL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9634092 A2 (GENENCOR INTERNATIONAL, INC.), 31 October 1996 (31.10.96), Fig 3A,B and the whole document	16-17
P,A	--	1-15
A	Dialog Information Services, File 55, BIOSIS PREVIEWS, Dialog accession no. 11434006, Biosis no. 98034006, Greenwood J M et al: "Purification and processing of cellulose-binding domain-alkaline phosphatase fusion proteins"; & Biotechnology and Bioengineering 44 (11). 1994. 1295-1305	16-17
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 July 1997	04 -07- 1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00042

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 110732 A1 (NOVO NORDISK A/S), 25 July 1991 (25.07.91) --	1-17
A	WO 9407998 A1 (NOVO NORDISK A/S), 14 April 1994 (14.04.94) --	1-17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00042

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next page

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

International application No.
PCT/DK 97/00042

According to rule 13.2, an international application shall relate to one invention only or a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over prior art.

The claimed invention relates to a process for removal or bleaching of soiling on cellulosic fabric using a modified enzyme which comprises a catalytically amino acid sequence of a non cellulolytic enzyme linked to an amino acid sequence comprising a cellulose-binding domain. SEQ ID No. 3 of claim 16 and SEQ ID No 4 of claim 17 encodes an enzyme having cellulolytic activity, thus the present claims lack unity, *a priori* .
The application claims the following inventions:

Invention 1, claims 1-17: A process and related compositions and enzyme hybrids, for removal or bleaching of soiling on cellulosic fabric using a modified enzyme which comprises a catalytically amino acid sequence of a non cellulolytic enzyme linked to an amino acid sequence comprising a cellulose-binding domain.

Invention 2, claims 16 and 17: An enzyme hybrid encoded by SEQ ID No 3 having the amino acid sequence of SEQ ID No 4.

An additional fee has been paid, thus, an additional search has been performed.

INTERNATIONAL SEARCH REPORT
Information on patent family members

03/06/97

International application No.
PCT/DK 97/00042

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9634092 A2	31/10/96	AU 5569296 A	18/11/96
		AU 5692796 A	18/11/96
		EP 0739982 A	30/10/96
		WO 9634108 A	31/10/96
WO 110732 A1	25/07/91	NONE	
WO 9407998 A1	14/04/94	EP 0663950 A	26/07/95
		FI 951629 A	05/04/95
		JP 8501692 T	27/02/96